(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 March 2003 (27.03.2003)

PCT

(10) International Publication Number
WO 03/024420 A1

(51) International Patent Classification[7]: A61K 9/00, 9/16, 9/51, 31/55, 31/50, A61P 27/02

(21) International Application Number: PCT/EP02/10314

(22) International Filing Date:
13 September 2002 (13.09.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0122318.9 14 September 2001 (14.09.2001) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LT, LU, LV, MA, MD, MK, MN, MX, NO, NZ, OM, PH, PL, PT, RO, RU, SE, SG, SI, SK, TJ, TM, TN, TR, TT, UA, US, UZ, VC, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: OPHTHALMIC DEPOT FORMULATIONS FOR PERIOCULAR OR SUBCONJUNCTIVAL ADMINISTRATION

(57) Abstract: The present invention relates to ophthalmic depot formulations comprising an active agent, e.g. embedded in a pharmacologically acceptable biocompatible polymer or a lipid encapsulating agent, e.g. for periocular or subconjunctival administration.

WO 03/024420 A1

OPTHALMIC DEPOT FORMULATIONS FOR PERIOCULAR OR SUBCONJUNCTIVAL ADMINISTRATION

The present invention relates to ophthalmic depot formulations for treatment of ocular diseases, in particular treatment of retinal and choroidal diseases.

Ocular diseases are difficult to treat as introduction of active agents into the eye and maintenance of therapeutically effective concentration thereof is difficult.

Oral administration of an active agent or parenteral administration of an active agent to a site other than the eye provides the active agent systemically. In order to achieve effective intraocular concentrations, systemic administration may necessitate administration of often unacceptably high levels of the active agent.

Injection of compositions comprising an active agent into the eye may be ineffective as the active agent may be washed out or is depleted from within the eye into the general circulation resulting in necessity for repeated administration, e.g. three injections in three to 42 days as described in US 5,632,984.

Introduction of slow release compositions, i.e. implants, into the eye, e.g. into an anterior segment or posterior segment of an eye as described in US 4,853,224, e.g. into the suprachoroidal space or pars plana of the eye as described in US 5,164,188, or e.g. into a site extrinsic to the vitreous comprising a suprachoroidal space, an avascular region of an eye, or a surgically-induced avascular region as described in US 5,824,072, by injection or surgical methods such as laser ablation, photocoagulation, cryotherapy, heat coagulation and the like is extremely painful and stressful for the patient. Implants may have to be removed when therapy is completed or no longer efficacious.
Applicants have found that ophthalmic depot formulations comprising an active agent may be administered, periocularly, e.g. retrobulbarly or sub-tenonly, or subconjunctivally.

Accordingly in one aspect, the present invention provides an ophthalmic depot formulation, comprising an active agent e.g. for periocular, e.g. retrobulbar or sub-tenon, or subconjunctival administration.

Ophthalmic depot formulations such as micro- or nanoparticle (hereinafter called microparticle) formulations, comprising an active agent e.g. embedded in a biocompatible pharmacologically acceptable polymer e.g. in an encapsulating polymeric matrix, or embedded in a lipid encapsulating agent have been found to be particularly suitable. The ophthalmic depot formulation may also comprise microparticles of essentially pure active agent, e.g. microparticles consisting of the active agent.

These microparticles have a high contact surface.

In one aspect, the present invention provides an ophthalmic depot formulation comprising microparticles of essentially pure active agent.

The microparticles of essentially pure active agent, e.g. microparticles consisting of the active agent, may be in amorphous or crystalline form e.g. with a particle size of 1 to 200 microns.

In another aspect, the present invention provides an ophthalmic depot formulation such as microparticles comprising an active agent, e.g. embedded in a biocompatible pharmacologically acceptable polymer or a lipid encapsulating agent.

The depot formulations, e.g. in particular microparticle formulations, of the present invention are adapted to release all or substantially all the active material over an extended period of time, e.g. several weeks up to 6 months. The matrix, e.g. polymer or lipid matrix, if present, is adapted to degrade sufficiently to be transported from the site of administration within one to 6 months after release of all or substantially all the active agent.

The polymer matrix of polymeric microparticles may be a synthetic or natural polymer. The polymer may be either a biodegradable or non-biodegradable or a combination of biodegradable and non-biodegradable polymers, preferably biodegradable.

Suitable polymers include

(a) linear or branched polyesters which are linear chains radiating from a polyol moiety, e.g. glucose,

(b) polyesters such as D-, L- or racemic polylactic acid, polyglycolic acid, polyhydroxybutyric acid, polycaprolactone, polyalkylene oxalate, polyalkylene glycol esters of acids of the Kreb's cycle, e.g. citric acid cycle, and the like and combinations thereof,

(c) polymers of organic ethers, anhydrides, amides, and orthoesters

(d) copolymers of organic esters, ethers, anhydrides, amides, and orthoesters by themselves or in combination with other monomers,

(e) polyvinylalcohol.

The polymers may be cross-linked or non-cross-linked, usually not more than 5%, typically less than 1%.

The desired rate of degradation of polymers and the desired release profile for active agent may be varied depending on the kind of monomer, whether a homo- or a copolymer or whether a mixture of polymers is employed.

The preferred polymers of this invention are linear polyesters, and branched chain polyesters. The linear polyesters may be prepared from the α-hydroxy carboxylic acids, e.g. lactic acid and glycolic acid, by the condensation of the lactone dimers, see e.g. US 3,773,919.

Linear polylactide-co-glycolides (PLG) which are preferably used conveniently have a molecular weight between 25,000 and 100,000 and a polydispersity M_w/M_n e.g. between 1.2 and 2.

The branched polyesters preferably used according to the invention may be prepared using polyhydroxy compounds e.g. polyol e.g. glucose or mannitol as the initiator. These esters of a polyol are known and described in GB 2,145,422 B. The polyol contains at least 3 hydroxy groups and has a molecular weight of up to 20,000, with at least 1, preferably at least 2, e.g. as a mean 3 of the hydroxy groups of the polyol being in the form of ester groups, which contain poly-lactide or co-poly-lactide chains. Typically 0.2% glucose is used to initiate polymerization. The branched polyesters (Glu-PLG) have a central glucose moiety having rays of linear polylactide chains, e.g. they have a star shaped structure. The preferred polyester chains in the linear and star polymer compounds preferably used according to the invention are copolymers of the alpha carboxylic acid moieties, lactic acid and glycolic acid,

or of the lactone dimers. The molar ratios of lactide: glycolide is from about 75:25 to 25:75, e.g. 60:40 to 40:60, with from 55:45 to 45:55, e.g. 55:45 to 50:50 the most preferred.

The branched polyesters having a central glucose moiety having rays of linear polylactide chains (Glu-PLG) may be prepared by reacting a polyol with a lactide and preferably also a glycolide at an elevated temperature in the presence of a catalyst, which makes a ring opening polymerization feasible.

The branched polyesters having a central glucose moiety having rays of linear polylactide chains (Glu-PLG) preferably have an average molecular weight M_n in the range of from about 10,000 to 200,000, preferably 25,000 to 100,000, especially 35,000 to 60,000 and a polydispersity e.g. of from 1.7 to 3.0, e.g. 2.0 to 2.5. The intrinsic viscosities of star polymers of M_n 35,000 and M_n 60,000 are 0.36 respectively 0.51 dl/g in chloroform. A star polymer having a M_n 52,000 has a viscosity of 0.475 dl/g in chloroform.

Suitable lipid encapsulating agents for lipid microparticles include phosphatidyl compounds such as phosphatidyl choline (PC), phosphatidyl serine (PS), and phosphatidyl ethanolamine (PE), sphingolipids, cerebrosides, ganglosides, steroids, e.g. cholesterol, etc.

The terms microsphere, microcapsule and microparticle are considered to be interchangeable with respect to the invention, and denote the encapsulation of the active agent by the polymer, preferably with the active agent distributed throughout the polymer, which is then a matrix for the active agent. In that case preferably the terms microsphere or more generally microparticle are used.

The microparticles, e.g. microspheres or microcapsules, may have a diameter from a few submicrons to a few millimeters, e.g. from about 0.01 microns to about 2 mm, e.g. from about 0.1 microns to about 500 microns. For pharmaceutical micro-particles, diameters of at most about 250 microns, e.g. 10 to 200 microns, preferably 10 to 130 microns, more preferably 10 to 90 microns, even more preferably 10 to 60 microns, are strived for, e.g. in order to facilitate passage through an injection needle.

Typically, the active agent will be from about 1 to 80, more usually 10 to 75% by weight of the polymeric microparticles and from 1 to 20% by weight of the lipid microparticles.

In another aspect, the present invention provides a liquid formulation, comprising a pharmaceutical acceptable polymer and a dissolved or dispersed active agent. Upon injection, the polymer forms a depot at the injection site, e.g. by gelifying or precipitating.

The depot formulations, in particular microparticle formulations, according to the present invention are suitable for the incorporation of a large variety of water soluble or hydrophobic active agents.

Active agents of particular interest include

i) anti-glaucoma drugs, such as the beta-blockers, e.g. timolol maleate, betaxolol, carteolol and metipranolol; epinephrine and prodrugs; such as dipivefrin; carbonic anhydrase inhibitors; such as dorzolamide, brinzolamide, acetazolamide, dichlorphenamide and methazolamide; dopaminergics, prostaglandins, docosanoids, alpha2 agonists; angiotensin II antagonists; alpha1 antagonists; cannabinoids; endothelin antagonists;

ii) miotics, e.g. pilocarpine, acetylcholine chloride, isoflurophate, demecarium bromide, echothiophate iodide, phospholine iodide, carbachol, and physostigmine;

iii) drugs for treatment of macular degeneration, such as interferon, particularly α-interferon; transforming growth factor (TGF), e.g. TGF-β;

iv) anti-cataract and anti-proliferative diabetic retinopathy (PDR) drugs, such as aldose reductase inhibitors: e.g. tolrestat, or angiotensin-converting enzyme inhibitors, e.g. lisinopril, enalapril;

v) drugs for treatment of age-related exudative macular degeneration (AMD), e.g. ocular neovascular disease, such as staurosporines, phthalazine derivatives;

vi) anti-clotting agents, such as tissue plasminogen activator, urokinase, and streptokinase;

vii) drugs for treatment of ocular inflammatory diseases such as cortico-steroids; e.g. prednisolone, triamcinolone, dexamethasone, fluocinolone, cortisone, prednisolone, fluorometholone and the like, non-steroidal anti-inflammatory drugs, such as ketorolac tromethamine, diclofenac sodium, indomethacin, flurbiprofen sodium, and suprofen;

viii) antibiotics, such as loridine (cephaloridine), chloramphenicol, clindamycin, amikacin, gentamicin, tobramycin, methicillin, lincomycin, oxacillin, penicillin, amphotericin B, polymyxin B, cephalosporin family, ampicillin, bacitracin, carbenicillin, cephalothin,

colistin, erythromycin, streptomycin, neomycin, sulfacetamide, vancomycin, silver nitrate, sulfisoxazole diolamine, quinolones, and tetracycline;

ix) anti-fungal or anti-viral agents, such as miconazole, ketoconazole, idoxuridine, trifluridine, vidarabine (adenine arabinoside), acyclovir (acycloguanosine), gancyclovir, foscarnet sodium, cidofovir, valacyclovir, famciclovirtrisulfapyrimidine-2, nystatin, flucytosine, natamycin, aromatic diamidines e.g. dihydroxystilbamidine and piperazine derivatives, e.g. diethylcarbamaine;

x) cycloplegics and mydriatic agents, such as atropine, cyclopentolate, scopolamine, homatropine tropicamide and phenylephrine;

xi) drugs for the treatment of ocular neurodegenerative diseases such as isopropyl unoprostone, glutamate receptor antagonists, e.g. memantine, caspase inhibitors, calcium antagonists, sodium channel blockers, NOS-2 inhibitors or neurotrophic factors, e.g. glial derived neurotrophic factor (GDNF) or ciliary neurotrophic factor (CNTF);

xii) peptide drugs such as calcitonin, lypressin or a somatostatin or analogues thereof,

xiii) anti-VEGF drugs;

xiv) phosphodiesterase inhibitors;

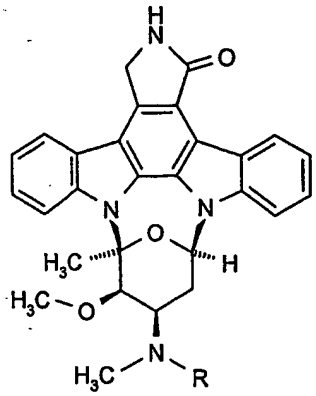
xv) antisense drugs such as fomivirsen sodium;

xvi) immunosuppressive agents; such as azathioprine, cyclosporin A, methotrexate, colchicine;

xvii) drugs for the treatment of ocular angiogenesis such as angiostatic steroids, PKC inhibitors, VEGF antagonists, COX2 inhibitors, ACE inhibitors or angiotensin II antagonists;

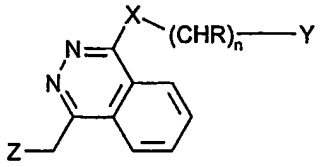
xviii) free radical scavengers, e.g. alpha tocopherol, carotenoids, sulfhydryl-containing compounds.

Preferably, active agents are drugs for treatment of the orbit region and ocular appendages, and for treatment of retinal and choroidal diseases comprising but not limited to age-related macular degeneration, diabetic retinopathy, glaucoma, inflammation, e.g. endophthalmitis, and bacterial, fungal or viral infections. Even more preferably, the active agent is a staurosporine of formula (I), a phthalazine of formula (II) or an ophthalmically acceptable salt thereof. Even more preferred are the staurosporine of formula (I) wherein R is benzoyl (hereinafter compound A), and the phthalazine of formula (II) wherein Z is 4-pyrididyl, X is imino, n is 0, and Y is 4-chlorophenyl (hereinafter compound B).



(I)

wherein
R is a hydrocarbyl radical R° or an acyl radical Ac



(II)

wherein
n is 0 to 2,
R is H or lower alkyl;
X is imino, oxa, or thia;
Y is aryl; and
Z is unsubstituted or substituted pyridyl, or an N-oxide of the defined compound, wherein one or more N atoms carry an oxygen atom

In another aspect, the present invention provides depot formulations and microparticles comprising a staurosporine of formula (I), a phthalazine of formula (II) or an ophthalmically acceptable salt thereof e.g. embedded in a biocompatible pharmacologically acceptable polymer, e.g. for periocular, e.g. retrobulbar or sub-tenon, or subconjunctival administration.

The microparticles of this invention may be prepared by any conventional technique, e.g. solvent evaporation, organic phase separation, spray drying, solvent extraction at low temperature or emulsion method, e.g. triple emulsion method. Using the phase separation or emulsion technique, the polymer is precipitated together with the drug, followed by hardening of the resulting product.

In another aspect, the present invention provides for a process for the production of microparticles comprising the steps of

a) dissolving the polymer or lipid encapsulating agent and the active agent in an organic solvent, e.g. methylene chloride,

b) mixing the solution of a) with an aqueous solution of polyvinyl alcohol (e.g. 0.5%). e.g. using a static mixer

c) collecting the generated microparticles, e.g. by a sedimentation, filtration or using a cyclon,

d) optionally washing of microparticles e.g. in a buffered solution of e.g. pH 3.0 to 8.0 or distilled water, and

e) drying under vacuo e.g. at a temperature of 20°C to 40°C.

The invention also relates to the microparticles prepared by this process.

The microparticles and the depot formulations of the present invention are useful for treatment of the known ophthalmic indications of the particular active agent incorporated therein. The utility of the formulations of the present invention may be observed in standard animal trials and clinical trials.

In a further aspect, the present invention provides a method for treating an ocular disease which comprises:

i) providing a depot formulation, e.g. a microparticle formulation, comprising an active agent e.g. embedded in a pharmacologically acceptable biocompatible polymer or a lipid encapsulating agent, and

ii) administering said depot formulation, e.g. microparticle formulation, periocularly, e.g. retrobulbarly or sub-tenonly, or subconjunctivally.

This method permits diffusion of said active agent from said depot formulation, e.g. a microparticle formulation, to the site of said ocular disease, e.g. the choroid, optic nerve, retina or vitreous. Preferably, the active agent is maintained at an effective dosage for said ocular disease at the site of said ocular disease for an extended period of time, e.g. for several weeks up to 6 months.

The depot formulations, e.g. microparticle formulations, may be administered, periocularly, e.g. retrobulbarly or sub-tenonly, or subjconjunctivally in a variety of ways including injection, trocar etc. Preferably, the active agent particles or the microparticles are suspended in a suitable liquid carrier.

The exact amount of active agent embedded in the polymer, i.e. the exact amount of depot formulation, e.g. microparticles formulation, to be administered depends on a number of

factors, e.g. the condition to be treated, the desired duration of treatment, the rate of release of active agent and the degradability of the polymeric matrix. The amount of active agent required may be determined on the basis of known in vitro or in vivo techniques. Repeated administration of the depot formulation of the invention may be effected when the polymeric matrix has sufficiently degraded.

Large amounts of active agent, e.g. up to 300 mg of active agent, e.g. in form of a suspension, may be administered in a single administration, e.g. in one injection. Frequency of dosing is variably dependent upon the severity of the syndrome. For severe cases dosing may occur once a month. The frequency is reduced when signs of the disease state show improvement. At that time dosing may be as infrequent as one dose every four or five months.

Filling may be effected before or after sterilization of the depot formulation. Sterilization of the formulation of the present invention and the primary package can be effected, e.g. by gamma irradiation e.g. at an energy of 25kGy, without degradation of active agent and/or microparticles.

Following is a description by way of example only of depot formulations of this invention.

Example 1 to 3: Preparations of microparticles

	Ex. 1	Ex. 2	Ex. 3
compound A	0.10 g	0.25 g	0.50 g
Glu-PLG	0.90 g	0.75 g	0.50 g
methylene chloride	2.5 ml	4.0 ml	9.5 ml
1.5% aq. polyvinyl alcohol	500 ml	600 ml	900 ml
0.5% aq. polyvinyl alcohol	3 l	3 l	3 l

Compound A and the polymer Glu-PLG are dissolved in the methylene chloride. The resulting solution is pumped through a static mixer together with a 1.5% solution of polyvinyl alcohol in water into a stirred solution of polyvinylalcohol in water (0.5%). The resulting suspension is heated to 42-48°C with stirring within 60 min and kept at that temperature for further 30 min before the mixture is cooled down to about 22°C within 50 min. The suspension is allowed to sediment for approximately 10 min. The aqueous solution of

polyvinyl is reduced under vacuo. The microparticles are washed with water for approximately 5 min. After sedimentation for 10 min, the solution is removed and the microparticles are filtered through an Ultipor filter, washed with water and dried under vacuo.

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Claims

1. An ophthalmic depot formulation comprising an active agent for periocular or subconjunctival administration.

2. A formulation according to claim 1 comprising of microparticles of essentially pure active agent.

3. A formulation according to claim 1 wherein the active agent is embedded in a biocompatible pharmacologically acceptable polymer or a lipid encapsulating agent.

4. A formulation according to claim 1 or 3 wherein the polymer is a polylactide-co-glycolide ester of a polyol.

5. A formulation according to any one of claims 1, 3 or 4 wherein the polymer is a 40/60 to 60/40 polylactide-co-glycolide ester of a polyol.

6. A formulation according to any one of claims 1, and 3 to 5 comprising microparticles.

7. A formulation according to claim 6 wherein the external surface of the microparticles is substantially free of active agent.

8. A liquid formulation comprising a dissolved pharmaceutical acceptable polymer and a dissolved or dispersed active agent which formulation upon injection forms a depot at the injection site.

9. A formulation according to any preceding claim wherein the active agent is present in an amount of up to 300 mg per dose for single administration.

10. A formulation according to any preceding claim wherein the active agent is a staurosporine of formula (I), a phthalazine of formula (II) or an ophthalmically acceptable salt thereof.

11. A method for treating an ocular disease which comprises:
    i) providing a depot formulation comprising an active agent, and

ii) introducing said depot formulation periocularly or subconjunctivally.

12. A method according to claim 11 wherein the active agent is embedded in a pharmacologically acceptable biocompatible polymer or a lipid encapsulating agent.

13. A method according to claim 11 or 12 wherein the active agent diffuses from said depot formulation to the site of said ocular disease.

14. A method according to any one of claims 11 to 13 wherein the active agent is maintained at an effective dosage for said ocular disease at the site of said ocular disease for an extended period of time.

15. A method according to any one of claims 11 to 14 wherein the active agent is maintained at an effective dosage for up to 3 months.

16. A microparticle comprising a staurosporine of formula (I), a phthalazine of formula (II) or an ophthalmically acceptable salt thereof embedded in a biocompatible pharmacologically acceptable polymer or a lipid encapsulating agent.

Country Application

Case Number: 99-216 **Country:** JP **SubCase:** KK4

Client: University of Illinois Japan

Title: Reagents And Methods For Identifying And Modulating Expression Of Tumor Senescence Genes

Case Type: PCT **Application Status:** Pending

Application Number: **Filing Date:** 24-Dec-2004

Publication Number: **Publication Date:**

Priority Number: **Priority Date:**

Patent Number: **Issue Date:**

Parent/PCT Number: US03/20425 **Parent/PCT Date :** 27-Jun-2003

Parent Issue Number: **Parent Issue Date :**

Tax Schedule: **Expiration Date:**

Confirmation #: **Patent Term Adjustment:** 0

File Out Date: 28-Dec-2004

File Out To: GMB

Agent: M&M **Client Reference #:**

Miyoshi & Miyoshi

Storage Location:

Family Reference: WO-KK/ORD

Remarks:

Assignee: **Working Atty:** KEN **Backup Atty:** JJD

User ID: garzelloni

Date Created: 27-Dec-2004

Last Update: 28-Dec-2004

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 June 2003 (05.06.2003)

PCT

(10) International Publication Number
WO 03/046185 A1

(51) International Patent Classification[7]: C12N 15/63, C07K 14/00, A61F 13/15

(21) International Application Number: PCT/US02/20565

(22) International Filing Date: 26 June 2002 (26.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/996,507 28 November 2001 (28.11.2001) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/046185 A1

(54) Title: POLYCATIONIC WATER SOLUBLE COPOLYMER AND METHOD FOR TRANSFERRING POLYANIONIC MACROMOLECULES ACROSS BIOLOGICAL BARRIERS

(57) Abstract: The invention presents plycation-grafted copolymers exhibiting substantial water solubility and low toxicity. The copolymers can be used to deliver drug and other therapeutic agents to specifically targeted cells.

POLYCATIONIC WATER SOLUBLE COPOLYMER AND METHOD FOR TRANSFERRING POLYANIONIC MACROMOLECULES ACROSS BIOLOGICAL BARRIERS

BACKGROUND OF THE INVENTION

1. The Field of the Invention

The present invention relates to the transport of biologically active agents across biological barriers. More specifically, the present invention relates to methods and compositions that enhance transport of polyanionic macromolecules such as DNA, RNA, antisense oligonucleotides and their analogs across biological barriers.

2. Technical Background

Gene therapy and antisense technology have been highly promoted for their potential to treat or cure a number of diseases. Many viral diseases and genetic conditions can potentially be treated by gene therapy. A great number of genes that play a role in previously untreatable diseases such as cancer, autoimmune diseases, cystic fibrosis and the like have been discovered. With the discovery of the gene involved, researchers have determined that the disease can be treated by either blocking a gene that is being over-expressed or by providing a copy of a malfunctioning gene. Often these treatments require the administration of DNA, RNA, antisense oligonucleotides, and their analogs to achieve a desired intracellular effect.

These treatment strategies have been shown to block the expression of a gene or to produce a needed protein in cell culture. However, a major problem with these promising treatments, is adapting them for use *in vivo*. For a compound to be an effective pharmaceutical agent *in vivo*, the compound must be readily deliverable to the patient, not rapidly cleared from the body, have a tolerable level of toxicity, and be able to reach the site within the body where it is needed.

However, macromolecules such as DNA, RNA, antisense oligonucleotides, and their analogs share similar, significant pharmaceutical problems. While these compounds are generally not toxic, if there are admistered orally, they do not reach the desired site because they are digested and metabolized. Injection of these polyanionic macromolecules increases the length of time the molecules are in the body, but does not target the specific area of need. Moreover they are subject to rapid degradation within the blood stream and clearance from the body.

Because DNA, RNA, and oligonucleotides are polyanionic macromolecules they do not readily cross biological barriers. The transfer of these materials into living cells is the major impediment to their use as therapeutic agents. An effective gene and oligonucleotide delivery system will need to bind to an appropriate cell, be internalized by endocytosis, escape from the lysosome and ultimately transfer the intact free DNA or oligonucleotides to

the nucleus or plasma. In another words, the success of gene therapy and antisense therapy is largely dependent on achieving a delivery of nucleic acids in sufficient quantities, to the correct target site of action, and for the desired time frame.

Many different strategies, including both viral and non-viral systems, have been attempted for the effective delivery of genes and oligonucleotides. Each of these strategies has had varying degrees of success. However, none of them are safe and efficient enough for clinical use. Toxicity, transfection efficiency, nucleic acid (NA) degradation and free NA release are challenging problems for all of the current non-viral gene delivery systems, including liposomes and cationic polymers.

A particular problem with non-viral delivery systems is the balance between the stability of the NA/carrier complex and the ability of the carrier to release the NA in the targeted cell. The NA/carrier complex must be stable enough to remain intact in the circulation system, but yet unstable enough to release the free NA at the target site.

One approach that has been used to allow entry of the polyanionic macromolecules to the cell cytoplasm is complexing the polyanionic macromolecule to a highly polycationic polymer such as PEI. PEI is a highly polycationic synthetic polymer. It has been used for years in common processes such as paper production, shampoo manufacturing, and water purification. Recently, PEI has become one of the most successful polycation carriers used in oligonucleotide and DNA delivery.

PEI has been shown to be a highly efficient carrier for delivering oligonucleotides and plasmids, both *in vitro* and *in vivo*. PEI is available in both linear and branched forms. Because of its high positive charge density, PEI spontaneously forms interpolyelectrolyte complex (Polyion complex) with nucleic acid as a result of cooperative electrostatic interaction between the ammonium groups of the PEI and the phosphate groups of the nucleic acid. The ability of PEI to transfect a wide variety of cells is well established. Compared to other polycationic carriers, PEI has proved to be much better in protecting against nucleic acid degradation and releasing the nucleic acid to the cytoplasm after endocytosis.

The transfection mechanism has been explored by different laboratories, but still is not quite clear. It is generally accepted that PEI transfection of cells begins with the entry of PEI via endocytosis. Then the complex or the PEI buffers the acidic pH of the lysosome, protecting the nucleic acid degradation and causing an osmotic swelling/rupture of the vesicles. The rupture of the vesicle releases the nucleic acid into the cytoplasm. The dissociation of free nucleic acid from the cationic polymer is generally assumed to be accelerated by the replacement of cellular polyanionic molecules. It is believed that protonation of the PEI leads to an expansion of the polymeric network due to the intramolecular charge repulsion.

However, PEI is not a perfect transfecting agent. For example, the PEI/NA complex usually produces serious aggregations in physiological buffers. Moreover, the complexes show limited stability in the presence of serum and are rapidly cleared out of the bloodstream following systemic administration. Moreover, PEI has been consistently observed to be toxic both *in vitro* and *in vivo*. These properties have significantly limited the biomedical applications of PEIs.

To partially overcome the toxic effects of the PEI and the aggregation problems of the PEI/NA complex in biological buffers, the polymer has been conjugated or grafted with both hydrophilic and hydrophobic groups. Grafting of the PEIs with PEG results in copolymers that can form relatively stable DNA complexes in aqueous buffers. However, transfection activity of these systems is much lower than that of unmodified PEI (25kDa). Partially propionyl acylated liner PEI (50kDa and 200 kDa) also shows less toxicity, but again this modification compromises the transfection activity. Conjugation of targeting groups, such as transferrin, mannose, and galactose, increased the transfection efficiencies toward targeted tissue, but still do not solve the intrinsic toxicity problems associated with high molecular PEIs, because high molecular PEIs have to be used as precursors in order to get efficient transfection activities. Small sized PEIs are much less toxic, but unfortunately low molecular weights PEIs (less than 2,000 Dalton) were found to produce no or very low transfection activities in various conditions.

In light of the foregoing, it would be an advancement in the art to provide a method of delivering polyanionic macromolecules to target cells. It would be an additional advancement to provide a carrier molecule that could efficiently transport the polyanionic macromolecules to across biological barriers. A further advancement would be achieved if the carrier molecule showed reduced toxicity as compared to presently available compounds. It would be a further advancement if the carrier/macromolecule complex were stable exhibited serum stability. It would be a further advancement if carrier/macromolecule complex could readily disassociate within the target cell. It would be a further advancement to provide a carrier molecule that could be targeted to a specific tissue or cell type.

BRIEF SUMMARY OF THE INVENTION

This invention provides a novel class of polycation grafted biocompatible copolymers which can be used as carrier molecules to deliver a polyanionic macromolecule to a cell. Two or more polycationic polymer fragments are covalently linked to a biocompatible

hydrophilic backbone polymer by linkers. The number of polycationic polymer fragments bound to the backbone polymer may be in the range from about 4 to about 100. It has been found that a number of polycationic fragments in the range from about 8 to about 15 can be successfully used to bind a polyanionic macromolecule and transfer the polyanionic macromolecule across biological barriers such as a cell wall or a plasma membrane. A variety of biocompatible polymers may be used as the backbone polymer. The backbone polymer may be, for example, polyethylene glycol (PEG), poly (N-(2-hydroxylpropyl)methacrylamide), or copolymers thereof. Likewise a variety of polycationic polymers may be linked to the backbone polymer. The polycationic polymer may be, for example, polyalkylamine (PAM), polyethylenimine (PEI), polylysine (PL), a polypeptide, chitosan, a polysaccharide, or copolymers thereof.

The carrier molecule may also include at least one targeting moiety connected to the biocompatible hydrophilic backbone or to the polycationic polymer. The targeting moiety can be selected to bind to a specific biological substance or site. Thus, the targeting moiety can be chosen based on its ability to bind to a molecule expressed in a specific cell type or specific tissue allowing the polyanionic macromolecule to be selectively delivered to the cell or tissue. Such targeting moieties may include a ligand, an antigen, a hapten, biotin, lectin, galactose, galactosamine, a protein, a histone, a polypeptide, a lipid, a carbohydrate, a vitamin, and a combination thereof.

The carrier molecule may also include at least one lysis agent connected to the biocompatible hydrophilic backbone or to the polycationic polymer. The lysis agent can be selected to break down a biological membrane such as a cell, endosomal, or nuclear membrane, thereby allowing the polyanionic macromolecule to be released into the cytoplasm or nucleus of the cell. Such lysis agents may include a viral peptide, a bacterial toxin, a lytic peptide, aleveolysin, bifermentolysin, boutulinolysin, capriciolysin, cereolysin O, chauveolysin, histolyticolysin O, pneumolysin, sealigerolysin, septicolysin O, sordellilysin, streptoslysin O, tenaolysin or thuringolysin O, and active fragments thereof.

As mentioned previously, the polycationic polymers are covalently linked to the biocompatible backbone polymer by linkers. The targeting moiety and the lysis agent may also be covalently linked to the backbone polymer by a linker. Such linkers can be a hydrocarbon chain, a PEG fragment, a polypeptide, a linear polymer containing an ester bond, a linear polymer containing an amide bond, a linear polymer containing a disulfide bond, a linear polymer containing a hydrozone bond, a linear polymer containing an oxime bond or a combination thereof. The linkers can be biodegradable peptides that can be broken by chemicals or enzymes to release the polycationic polymer, the targeting moiety, or the lysis agent from the backbone polymer. Examples of such biodegradable peptide are GlyPheLeuGly (SEQ. ID. NO.: 1) and GlyPhePheGly (SEQ ID. NO.: 2). The linkers can

have a length from about 2 to about 100 atoms. Linkers with a length of about 3 atoms to about 30 atoms can also be used.

The biocompatible hydrophilic backbone can have a molecular weight that is selected to optimize the delivery of the polyanionic macromolecule to the cell. Thus, in certain embodiments the backbone polymer has a molecular weight in the range from about 1,000 to about 1,000,000. A backbone polymer with a molecular weight in the range from about 5,000 to about 100,000 may also be used. A biocompatible hydrophilic backbone with a molecular weight of about 20,000 to about 40,000 can be used to deliver the polyanionic macromolecule to the cell.

The molecular weight of the polycationic polymers can also be selected for optimal delivery of the polyanionic macromolecule to the target cell. The molecular weight can be in the range from about 100 about 100,000. Alternatively the molecular weight of the polycationic polymers can be in the range from about 200 to about 10,000. A polycationic polymer with a molecular weight in the range from about 400 to about 2,000 can be used to deliver the polyanionic macromolecule to the cell.

The present invention also relates to a complex for delivering a polyanionic macromolecule to a cell. The complex may have a carrier molecule as described above complexed with a polyanionic macromolecule. The complex may be given to an animal *in vivo* or a cell culture. The complex allows the polyanionic macromolecule to be delivered to the a cell within the animal or cell culture.

The polyanionic macromolecule can be selected from a number of macromolecules that are useful in the treatment of disease or in laboratory experimentation. In certain configurations of the complex, the polyanionic macromolecule is a nucleic acid such as RNA, DNA, or a combination or derivative thereof. The nucleic acid can be, for example, genomic DNA, plasmid DNA, synthetic DNA, or RNA. Other types of nucleic acids that can be used with the carrier molecule of present invention are, for example, an antisense oligonucleotide, ribozyme, DNAzyme, chimeric RNA/DNA oligonucleotide, phosphorothioate oligonucleotide, 2'-O-methyl oligonucleotides, DNA-PNA conjugates, DNA-morpholino-DNA conjugates, and combinations thereof.

The invention also provides a method of transporting a polyanionic macromolecule across a biological barrier of a cell. The biological barrier can be a cell wall, a plasma membrane, or like cell membrane. The cell may be for example a cell in a cell culture. Alternatively the cell may be a cell within a multicellular organism such as a plant or an animal. The cell can be a cell derived from an organism such as hepatocytes, liver cells, kidney cells, brain cells, bone marrow cells, nerve cells, heart cells, spleen cells, stem cells and co-cultures of the above. Moreover, the cells may be from established cell lines such a HepG Hep G2 and Hela cells. The method of transporting the polyanionic macromolecule across the barrier includes complexing the polyanionic macromolecule to a carrier molecule

of the present invention to create a complex. The cell is then contacted with the carrier molecule to deliver the polyanionic macromolecule to the cell. The complex is then taken into the cell by, for example, endocytocis and then released into the cell cytoplasm.

BRIEF DESCRIPTION OF THE DRAWINGS

A more particular description of the invention briefly described above will be rendered by reference to specific embodiments thereof which are illustrated in the appended drawings. These drawings depict only typical embodiments of the invention and are not therefore to be considered to be limiting of its scope. The invention will be described and explained with additional specificity and detail through the use of the accompanying drawings in which:

Figure 1A is a schematic representation of the synthesis of one embodiment of a polycation grafted biocompatible copolymer of the present invention.

Figure 1B is a schematic representation of the synthesis of another embodiment of a polycation grafted biocompatible copolymer of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention provides a novel class of polycation grafted biocompatible copolymers which can be used as carrier molecules to deliver a polyanionic macromolecule to a cell. Two or more polycationic polymer fragments are randomly covalently linked to a biocompatible hydrophilic backbone polymer by a linker. The number of polycationic polymer fragments bound to the backbone polymer may be in the range from about 4 to about 100. It has been found that a number of polycationic fragments in the range from about 8 to about 15 can be successfully used to bind a polyanionic macromolecule and transfer the polyanionic macromolecule across biological barriers. As used herein biocompatible refers to a substance that has limited immunogenic and allergenic ability. Biocompatible also means that the substances does not cause significant undesired physiological reactions. A biocompatible substance may be biodegradable. As used herein biodegradable means that a substance such as the backbone polymer or the polycationic polymer can chemically or enzymatically break down or degrade within the body. A biodegradable substance may form nontoxic components when it is broken down. Moreover, the biocompatible substance can be biologically neutral, meaning that it lacks specific binding properties or biorecognition properties.

A variety of biocompatible polymers may be used as the backbone polymer. The backbone polymer may be, for example, polyethylene glycol (PEG), poly (N-(2-hydroxylpropyl)methacrylamide), or copolymers thereof. Likewise a variety of polycationic polymers may be linked to the backbone polymer. The polycationic polymer may be, for

example, polyalkylamine (PAM), polyethylenimine (PEI), polylysine (PL), a polypeptide, chitosan, a polysaccharide, or copolymers thereof.

PEG has many qualities that make it a desirable biocompatible backbone polymer for use with the carrier polymers of the invention. First, PEG is commercially available in a variety of molecular masses at low dispersity (Mw/Mn< 1.1). Based on their molecular size, PEG polymers are arbitrarily classified into low molecular weight PEG (Mw<20,000) and high molecular weight PEG (Mw>20,000). A recent study found that the renal clearance of PEG decreased with an increase in molecular weight, with the most dramatic change occurring at a MW of 30,000 after intravenous administration. The halftime (tl/2) of PEG circulating in blood also showed a concomitant and dramatic increase. For instance, the tl/2 for PEG went from approximately 18 min to 16.5 hour as the molecular weight increased from 6,000 to 50,000. Consequently, conjugation of anticancer drugs with PEG of a molecular weight of 20,000 or greater can prevent rapid elimination of the PEG-conjugated species and allow for passive tumor accumulation.

The carrier molecule may also include at least one targeting moiety connected to the biocompatible hydrophilic backbone or to a bound polycationic polymer. The targeting moiety can be selected to bind to a specific biological substance or site herein referred to as the receptor. Thus, the targeting moiety can be chosen based on its ability to bind to a receptor molecule expressed in a specific cell type or specific tissue allowing the polyanionic macromolecule to be selectively delivered to the cell or tissue. The targeting moiety can be any signal member which is recognizable by a cell membrane receptor. Thus, in certain embodiments, the targeting moiety is a galtactose containing saccharide which specifically binds to liver cells or hepatoma cells. The galactose containing sacchride can be selected from the group consisting of lactose and galactose.

A targeting moiety refers to those moieties that bind to a specific biological substance or site. The biological substance or site is considered the target of the targeting moiety that binds to it. Ligands are one type of targeting moiety. Ligands have a selective (or specific) affinity for another substance known as the receptor. Because the ligand has a specific affinity for the receptor, the ligand binds to the receptor selectively over other molecules. Thus, when a ligand is used in conjunction with the carrier polymer of the present invention, the carrier polymer can be designed to bind to a receptor on a specific cell type. This selective binding allows for the selective delivery of the polyanionic macromolecule to the target cell. Examples of ligands suitable for targeting cells are antigens, haptens, biotin, biotin derivatives, lectins, galactose, galactosamine vitamin and fucosylamine moieties, receptors, substrates, coenzymes and cofactors among others.

When applied to the polycation grafted copolymers of this invention, a ligand includes an antigen or hapten that is capable of being bound by, or to, its corresponding antibody or an active fraction thereof. Also included are viral antigens or hemagglutinins and

neuraminidases and nucleocapsids including those from any DNA viruses, RNA viruses, HIV, hepatitis viruses, adenoviruses, alphaviruses, arenaviruses, coronaviruses, flaviviruses, herpesviruses, myxoviruses, oncornaviruses, papovaviruses, paramyxoviruses, parvoviruses, picornaviruses, poxviruses, reoviruses, rhabdoviruses, rhinoviruses, togaviruses, and viriods. The ligand may be selected from any bacterial antigens including those of gram-negative and gram-positive bacteria, acinetobacter, achromobacter, bacteroides, clostridium, chlamydia, enterobacteria, haemophilus, lactobacillus, neisseria, staphyloccus, and streptoccocus. Other suitable ligands include any fungal antigens such as those of aspergillus, candida, coccidiodes, mycoses, phycomycetes, and yeasts. Other antigens such as mycoplasma antigens, rickettsial antigens, protozoan antigens, and parasite antigens are suitable ligands in certain embodiments of the invention. Human antigens including those of blood cells, virus infected cells, genetic markers, oncoproteins, plasma proteins, complement factors, alphafetoprotein, prostate specific antigen (PSA), cancer markers, and rheumatoid factors may also serve as suitable ligands.

There are many other substances that may be used as appropirate ligands to direct the carrier copolymer to the target cell. Among these substances are proteins, histones, hormones, vitamins, steroids, prostaglandins, synthetic or natural polypeptides, carbohydrates, lipids, antibiotics, drugs, digoxins, pesticides, narcotics, and neurotransmitters. Ligands also refers to various substances with selective affinity for a that are produced through recombinant DNA, genetic and molecular engineering.

The receptor for a ligand is an important consideration in selecting a ligand to target a cell. The receptor may also be referred to as a ligator, binding body, or a binding partner. The receptor functions as a type of biorecognition molecule that selectivley binds to the ligand. The receptor is molecule that is generally, but not necessarily, larger than the ligand that binds it. A receptor can be a protein such as an antibody or a non-protein binding body. As used herein an antibody refers to all classes of antibodies including monoclonal antibodies, chimeric antibodies, Fab fractions, and derivatives thereof. Other receptors suitable for targeting include naturally occurring receptors, hemagglutinins, and cell membrane and nuclear derivatives that bind specifically to hormones, vitamins, drugs, antibiotics, cancer markers, genetic markers, viruses, and histocompatibility markers. Another group of receptors includes RNA and DNA binding proteins. Other potentially useful receptors for targeting are cell surface enzymes such as neuraminidases, plasma proteins, avidins, streptavidins, chalones, cavitands, thyroglobulin, intrinsic factor, globulins, chelators, surfactants, organometallic substances, staphylococcal protein A, protein G, ribosomes, bacteriophages, cytochromes, lectins, certain resins, and organic polymers. Receptors also include various substances such as any proteins with selective affinity for ligands that are produced through recombinant DNA and genetic and molecular engineering.

The carrier molecule may also include at least one lysis agent connected to the biocompatible hydrophilic backbone or to a bound polycationic polymer. The lysis agent could be any membrane fusion peptide or protein. The lysis agent can be selected to break down a biological membrane such as a cell, endosomal, or nuclear membrane, thereby allowing the polyanionic macromolecule to be released into the cytoplasm or nucleus of the cell. As a result of the presence of the lysis agent, the membrane undergoes lysis, fusion, or both. Such lysis agents may include a viral peptide, a bacterial toxin, a lytic peptide, aleveolysin, bifermentolysin, boutulinolysin, capriciolysin, cereolysin O, chauveolysin, histolyticolysin O, pneumolysin, sealigerolysin, septicolysin O, sordellilysin, streptoslysin O, tenaolysin or thuringolysin O, and active fragments thereof. A lytic peptide is a chemical grouping which penetrates a membrane such that the structural organization and integrity of the membrane is lost. Lysis agent also include viruses and synthetic compounds that can break down a biological membrane. Fragments of the above listed lysis agents which will provide endosomal escape activity may also be employed in the present invention. Other peptides and proteins are known to cause the breakdown or fusion or biological membranes and may be used as a lysis agent within the scope of the invention. Jahn, R. & Sudhof, T., *Annu. Rev Biochem* **68**: 863-911(1999); Pecheur, E.I., et al, *J Membrane Biol.* **167**: 1-17 (1999).

As mentioned previously, the polycationic polymer is covalently linked to the biocompatible backbone polymer by a linker. The targeting moiety and the lysis agent may also be covalently linked to the backbone polymer or the bound polycationic polymer by a linker. Such linkers can be a hydrocarbon chain, a PEG fragment, a polypeptide, a linear polymer containing an ester bond, a linear polymer containing an amide bond, a linear polymer containing a disulfide bond, a linear polymer containing a hydrozone bond, a linear polymer containing an oxime bond or a combination thereof. The linkers may either be biodegradable linkers or non-biodegradable linkers. Examples of biodegradable linkers are short peptides and disulfide linkers (-(CH_2)xSS(CH_2)x- wherein x is an integer of 2 to 8). Non-biodegradable linkers include hydrocarbon linkers such as -(CH_2)n- or -(CH_2CH_2O)n- where n is an integer of 2 to 50.The linkers can have a length from about 2 to about 100 atoms. Linkers with a length of about 3 atoms to about 30 atoms can also be used.

The linkers used to covalently link the polycationic polymer to the backbone polymer can be configured to allow for the controlled release complexed polyanionic macromolecule from the carrier. Controlled release indicates that the nucleic acid or other polyanionic macromolecule is released from the copolymer carrier complex only by cleavage of the linker that were used to synthesize the carrier. Thus, controlled release does not include the release of the polyanionic macromolecule by diffusion until the linkages are cleaved.

Biodegradable linkers include, but are not limited to, two categories of bonds. A first category includes disulfide linkages and ester bonds. Disulfide linkages and ester bonds are

known for covalent coupling of pharmaceutical compounds to polymers. However, this category of bonds has limited value for delivering pharmaceutical compounds *in vivo* because these bonds are subject to cleavage in the blood stream. The second category includes bonds that are generally cleaved after entering the cell (intracellular cleavage). This category of linkers are cleavable under acidic conditions like those found in lysosomes or by enzymes thereby allowing the pharmaceutical compound to be released intracellularly.

The bonds that are cleaved under acidic conditions are known as acid-sensitive or acid-liable bonds. One example of an acidsensitive bond is a hydrazone linkage. Greenfield, et al, *Cancer Res.* **50**: 6600-6607 (1990). Enzyme-sensitive linkers include polypeptides that contain amino acid sequences that make the polypeptide hydrophobic. These polypeptides are cleaved by specific enzymes such as cathepsins, found primarily inside the cell. Such polypeptides can be synthetic or naturally occurring peptides. Examples of suitable biodegradable polypeptide linkers are GlyPheLeuGly (SEQ. ID. NO.: 1) and GlyPhePheGly (SEQ. ID. NO.: 2). Another type of biodegradable linkage is a "hindered" or "protected" disulfide bond that sterically inhibits attack from thiolate ions. Such protected disulfide bonds are found in the coupling agents S-4-succinimidyloxycarbonyl-.alpha-.methyl benzyl thiosulfate (SMBT) and 4-succinimidyloxycarbonyl-.alpha-.methyl-.alpha.-(2-pyridyldithio) toluene (SMPT).

The biocompatible hydrophilic backbone can have a molecular weight that is selected to optimize the delivery of the polyanionic macromolecule to the cell. Thus, in certain embodiments the backbone polymer has a molecular weight in the range from about 1,000 to about 1,000,000. A backbone polymer with a molecular weight in the range from about 5,000 to about 100,000 may also be used. A biocompatible hydrophilic backbone with a molecular weight of about 20,000 to about 40,000 can be used to deliver the polyanionic macromolecule to the cell.

The molecular weight of the polycationic polymer can also be selected for optimal delivery of the polyanionic macromolecule to the target cell. The molecular weight can be in the range from about 100 about 100,000. Alternatively the molecular weight of the polycationic polymer can be in the range from about 200 to about 10,000. A polycationic polymer with a molecular weight in the range from about 400 to about 2,000 can be used to deliver the polyanionic macromolecule to the cell.

The present invention also relates to a complex for delivering a polyanionic macromolecule to a cell. Once the complex is delivered to the cell, the carrier molecule allows the complex to cross the cell wall and other biological barriers and gain access to the interior of the cell. The complex may have a carrier molecule as described above complexed with a polyanionic macromolecule. The complex may be given to an animal *in*

vivo or to a cell in culture. The complex allows the polyanionic macromolecule to be delivered to the a cell within the animal or cell culture.

The polyanionic macromolecule can be selected from a number of macromolecules that are useful in the treatment of disease or in laboratory experimentation. In certain configurations of the complex, the polyanionic macromolecule is a nucleic acid such as RNA, DNA, or a combination or derivative thereof. The nucleic acid can be, for example, genomic DNA, plasmid DNA, synthetic DNA, or RNA. Other types of nucleic acids that can be used with the carrier molecule of present invention are, for example, an antisense oligonucleotide, ribozyme, DNAzyme, chimeric RNA/DNA oligonucleotide, phosphorothioate oligonucleotide, 2'-O-methyl oligonucleotides, DNA-PNA conjugates, DNA-morpholino-DNA conjugates, and combinations thereof.

The invention also provides a method of transporting a polyanionic macromolecule across the biological barriers of the cell. The cell may be for example a cell in a cell culture. Alternatively the cell may be a cell within a multicellular organism such as a plant or an animal. The cell can be a cell derived from an organism such as hepatocytes, liver cells, kidney cells, brain cells, bone marrow cells, nerve cells, heart cells, spleen cells, stem cells and co-cultures of the above. Moreover, the cells may be from established cell lines such a HepG Hep G2 and Hela cells.

The method of delivering the polyanionic macromolecule to the cell includes complexing the polyanionic macromolecule to a carrier molecule of the present invention to create a complex. The cell is then contacted with the complexed carrier molecule to deliver the polyanionic macromolecule to the cell. The carrier complex may enter the cell by endocytocis and then escape from the vesicles to gain access to the cytoplasm of the cell. If the target cell is within a cell culture *in vitro*, the cell can be contacted with the complexed carrier molecule by providing the cells with a growth medium containing the polyanionic macromolecule/carrier complex or by inserting a solution containing the polyanionic macromolecule/carrier complex into the growth media. If the target cell is within an organism *in vivo*, the contacting may occur by positioning the complex within the organism so that it has access to the target cell. For example, the complex may be administered by injecting a solution containing the complex into the circulatory system of the organism. A carrier molecule with a targeting moiety attached will allow the complex to be directed to a target cell with a target corresponding to the targeting moiety. The polyainonic macromolecule/carrier complex may be administered to an organism by intramuscular, intraperitoneal, intraabdominal, subcutaneous, intravenous, and intraarterial delivery. Other methods of administration of the complex include parenteral, topical, transdermal, transmucosal, inhaled, and insertion into a body cavity such as by ocular, vaginal, buccal, transurethral, rectal, nasal, oral, pulmonary, and aural administration.

When the polymeric carrier molecules of the invention are complex with a nucleic acid or other drugs, they form polymeric micelles. Following intravenous administration, such polymeric micelles have been found to have a prolonged systemic circulation time. This prolonged circulation time is due to their small size and hydrophilic shell which minimize uptake by the mononuclear phagocyte system and to their high molecular weight which prevents renal excretion. Polymeric micelle incorporated drugs may accumulate in tumors to a greater extent than the free drug and show reduced distribution into untargeted areas such as the heart. Accumulation of polymeric micelles in malignant or inflamed tissues may be due to increased vascular permeability and impaired lymphatic drainage. The tumor vessels are more leaky and less permselective than normal vessels. Several *in vivo* studies have shown that polymeric micelles are able to improve the efficiency of anticancer drugs against leukemia and solid tumors. The studies indicated that PEG does not exhibit specific affinity for any organ and that its accumulation in tumor tissue is mainly governed by the level of hyperpermeable tumor vasculature (enhanced permeability retention or EPR effect), irrespective of the molecular mass of the polymer and the tumor loading site.

The EPR effect is considered as a passive targeting method, but drug targeting could be further increased by binding to targeting moieties such as antibodies or sugars or by introducing a polymer sensitive to variation in temperature or pH. Targeting micelles or pH sensitive micelles can serve for the delivery of drug to tumors, inflamed tissues or endosomal compartments, since they all are associated with a lower pH than normal tissue.

A solution of the grafted copolymer that contains nucleic acid or other polyanionic macromolecules can administered to the cultured cells or the body. An important consideration in the usefulness of a carrier molecules is how much drug can be loaded into the carrier. The molar ratio of the nitrogen on the carrier copolymer to the phosphate on the nucleic acid (the N/P ratio) should be considered. In most instances the N/P ratio in the complexes of the carrier polymer and nucleic acid molecules will be in the range of about 1 to about 50. More specifically, it is anticipated that for most uses the N/P ratio in the complexes will be in the range between about 2 to about 30. These ranges given above are not exclusive of the N/P ratio that may be used with the invention. As long as functionality is maintained, drug loadings outside of these ranges falls within the scope of the invention.

Referring to Figure 1A, the general synthesis of a carrier copolymer of the present invention is illustrated. Polyethylene glycol (PEG) of mean molecular weight is obtained. The PEG has a number "m" of pendant propionic acid groups (PA) randomly grafted onto its backbone. PEG-mPA and anhydrous dichloromethane are combined with the protection of argon. Then p-nitrophenol and 4-dimethylaminopyridine (DMAP) are added to the solution. Then 1-[3-dimethylaminopropyl]-3-ethylcarbodiimide hydrochloride (EDC) is added to form a clear solution. Then acetic acid is added to the clear mixture. The clear reaction mixture is then mixed with a solution of polyethylenimine (PEI) in anhydrous

dimethylformamide (DMF) under the protection of argon. The mixture may be concentrated on a rotary evaporator to remove most of the DMF solvent. The resulting product can be purified and concentrated to produce a wax product. The crude wax product can be further purified on a gel filtration column to yield purified PEG-mPA-PEI.

Referring to Figure 1B, the general synthesis of another carrier polymer of the present invention is illustrated. This carrier polymer is formed from a PEG backbone conjugated to PEI via a biodegradable polypeptide linker, GFLG. PEG-mPA is obtained as a starting material. The PEG-mPA is then converted to PEG-mPA-ONp. PEG-mPA-ONp is synthesized by dissolving PEG-mPA in anhydrous dichloromethane. Then p-nitrophenol and 4-dimethylaminopyridine (DMAP) are added. Then 1-[dimethylaminopropyl]-3-ethylcarbodiimide hydrochloride (EDC) is added. Next acetic acid is added to the solution. Then p-Toluenesulfonic acid monohydrate is added to neutralize the DMAP catalyst. The reaction yields a white product that is PEG-mPA-ONp.

The PEG-mPA-ONp product and GFLG tetrapeptide are then dissolved in anhydrous DMF. N,N-diisopropylethylamine (DIPEA) is added to this solution. The reaction mixture can be concentrated to remove excess solvent. Cold ethyl ether may be added to precipitate the product. The PEG-mPA-GFLG product is then purified. The PEG-mPA-GFLG product is reacted with polyethylenimine to form PEG-mPA-GLFG-PEI.

EXAMPLES

The following examples are given to illustrate various embodiments which have been made within the scope of the present invention. The following examples are neither comprehensive nor exhaustive of the many types of embodiments which can be prepared in accordance with the present invention.

Materials and General Methods

PEG with pendant propionic acid groups (PEG-8PA PEG-10PA, and PEG-15PA, Mw = ~20 KD, SunBio, Inc., Anyang City, South Korea) was dried overnight in vacuo at room temperature. PEI600 (Mw = 600), PEI1200 (Mw = 1,200), PEI2K (Mw = 1,800) and PEI10K (Mw = 10,000) were from Polysciences, Inc. of Warrington, PA. PEI400 (Mn = 423), PEI800 (Mw = 800) and PEI 25K (Mw = 25,000) were purchased from Aldrich Chemical Company, Inc. of Milwaukee, WI. Other chemicals were from Aldrich or VVR and used as received without further purification. HPLC analysis was performed on a Waters system equipped with Waters RI detector and Phenomenex Polysep-GPC-P 3000 column. ^{1}H-NMR was recorded on a Varian 400 MHz machine.

Example 1 - Synthesis of PEG20K-15PA-PEI400 (15 PEI400 grafted PEG-20K)

A dry 50 ml one necked flask was charged with 1.3 g of polyethylene glycol of mean molecular weight of about 20,000 with 15 pendant propionic acid groups (PEG20K-15PA) (~0.75 mmole pendant -COOH, dried overnight in vacuum in P_2O_5 desiccator) and 10 ml anhydrous dichloromethane with the protection of argon. About 0.15 g (1.1 mmoles) of p-nitrophenol and about 0.015 g of 4-dimethylaminopyridine were added to the flask. The mixture was stirred at room temperature to form a clear solution. Then about 0.20 g (1.0 mmoles)of fine powdered 1-[3-dimethylaminopropyl]-3-ethylcarbodiimide hydrochloride (EDC) was added in one portion. The mixture was again stirred for about 2 hours at room temperature following the dissolution of EDC. Then about 0.18 ml (3.2 mmoles) of acetic acid was added to the clear mixture. The mixture was stirred for an additional 30 minutes at room temperature. The clear reaction mixture was mixed with a solution of 20 ml of linear PEI 400 (Aldrich 46,853-3, Mn = ~423) in 20 ml anhydrous dimethylformamide (DMF) with vigorous stirring under protection of argon. The mixture was stirred at room temperature for about 4 hours, then concentrated on a rotary evaporator to remove most of the DMF solvent. The oil mixture was then diluted with water and purified on a gel filtration column (Sephacryl S-100, 2.5 X 90 cm). The desired copolymer fractions were pooled together after HPLC analysis. About 1.5 g of pure product was obtained. ^{1}H-NMR analysis indicated that the copolymer contains about 10% (w/w) PEI, indicating that the average molecular weight of the copolymer was about 23,444 assuming the average molecular weight of the starting PEG15PA is 20,000. ^{1}H-NMR (D_2O, 400 MHz), • 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 2.4-3.2 (m, 12, -CH_2CH_2N- of PEI).

Example 2 - Synthesis of PEG20K-15PA-PEI800 (15 PEI 800 grafted PEG20K)

Following the procedure of Example 1, 1.0 g of polyethylene glycol of mean molecular weight of about 20,000 with about 15 pendant propionic acid groups (PEG20K-15PA) reacted with polyethylenimine of mean molecular weight of about 800 (PEI800, 20 grams) to produce about 1.1 grams of PEI20K-15PA-PEI800. ^{1}H-NMR analysis indicates that the copolymer contains about 30% (w/w) PEI, indicating that the average molecular weight of the copolymer is about 28,400, assuming the average molecular weight of the starting PEG15PA is 20,000. ^{1}H-NMR (D_2O, 400 MHz), • 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 2.4-3.2 (m, 43.0, -CH_2CH_2N- of PEI).

Example 3 - Synthesis of PEG20K-8PA-PEI800 (8 PEI 800 grafted PEG-20K)

Following the procedure of Example 1, 1.0 gram of Polyethylene glycol of mean molecular weight of about 20,000 with about 8 pendant propionic acid groups (PEG20K-8PA) reacted with polyethylenimine of mean molecular weight of about 800 (PEI800, 20 grams) to produce about 1.2 grams of PEI20K-8PA-PEI800. ^{1}H-NMR analysis indicates

that the copolymer contains about 11.5% (w/w) PEI, which indicating that the average molecular weight of the copolymer is about 22,607, assuming the average molecular weight of the starting PEG-8PA is 20,000. ^{1}H-NMR (D_2O, 400 MHz), • 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 2.4-3.2 (m, 13.3, -CH_2CH_2N- of PEI).

Example 4: Synthesis of PEG-10PA-PEI1200 (10 PEI1200 grafted PEG20K)

A dry 1000 ml one neck flask was charged with 5.0 grams of PEG-10PA (mean molecular weight of about 20,000 with 10 pendant propionic acid groups, dried in P_2O_5 desiccator overnight), 0.56 grams of p-nitrophenol and 50 ml of anhydrous pyridine with the protection of argon. To the clear mixture was added 0.77 grams of 1-[dimethylaminopropyl]-3-ethyl carbodiimide (EDC). The mixture was stirred at room temperature for about 5 hours. Acetic acid (0.6 ml) was added with another 30 minute stirring at room temperature. The mixture was reacted with 100 ml of PEI1200 (Mw = 1,200) in 200 ml of anhydrous pyridine overnight at room temperature. The mixture was concentrated to remove the pyridine solvent on a rotary evaporator. The viscous solution was diluted to about 1000 ml with deion water. The solution was ultrafiltrated to about 60 ml followed by diafiltration with 2000 ml of deion water on a Pall Filtron Minim Diafiltration system equipped with a Memrane Centramate with a10K NMWC membrane cassette (Pall Corporation, East Hills, NY). The final product solution was concentrated on a rotary evaporator, about 4.5 grams of wax solid was obtained. The wax product was further purified by ether precipitation from methanol twice, about 4.1 grams of white powered PEG-10PA-PEI1200 was obtained. ^{1}H-NMR analysis indicates that the copolymer contains about 20% (w/w) PEI, indicating that the average molecular weight of the co-polymer is about 24,963, assuming the average molecular weight of the starting PEG-10PA is 20,000 Dalton. ^{1}H-NMR (D_2O, 400 MHz), • 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 2.4-3.2 (m, 29, -CH_2CH_2N- of PEI).

Example 5 - Synthesis of PEG20K-8PA-PEI2K (8 PEI1800 grafted PEG-20K)

Following the procedure of Example 1, 1.0 gram of Polyethylene glycol of mean molecular weight of about 20,000 with about 8 pendant propionic acid groups (PEG20K-8PA) was reacted with polyethylenimine of mean molecular weight of about 1,800 (PEI2K, about 20 grams) to produce about 1.1 grams of PEG20K-8PA-PEI2K. ^{1}H-NMR analysis indicates that the copolymer contains about 27% (w/w) PEI, indicating that the average molecular weight of the copolymer is about 27,490, assuming the average molecular weight of the starting PEG-8PA is 20,000. ^{1}H-NMR (D_2O, 400 MHz), • 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 2.4-3.2 (m, 38.3, -CH_2CH_2N- of PEI).

Example 6 - Synthesis of PEG20K-15PA-GFLG-PEI400 (15 PEI400 grafted PEG20K with GFLG linkers)

Referring now to Figure 1B, PEG20K-15PA-GLFG-PEI400 was synthesized according to the illustrated scheme. PEG20K-15PA-ONp was synthesized by dissolving polyethylene glycol (PEG) of mean molecular weight of about 20,000 with about 15 pendant propionic acid groups (PEG20K-15PA) (2.0 g, ~1.5 mmole -COOH,) in 20 ml anhydrous dichloromethane. Then about 292 mg (2.1 mmoles, Fw = 139) p-nitrophenol and about 26 mg (0.2 mmole) 4-dimethylaminopyridine (DMAP) were added to the solution. The mixture was stirred at room temperature to form a clear solution. Then about 402 mg (2.1 mmoles) of fine powdered 1-[dimethylaminopropyl]-3-ethylcarbodiimide hydrochloride (EDC) were added. The mixture was stirred at room temperature for about 3 hours. Next, about 0.4 ml of acetic acid was added, and the mixture was stirred for an additional 30 minutes. About 400 mg (2.1 mmoles, Fw=190.22) p-Toluenesulfonic acid monohydrate was added to neutralize the DMAP catalyst. The mixture was stirred at room temperature until all solids were dissolved. About 40 ml isopropanol was added to the solution. Then approximately 20 ml of solvent was removed in vacuum on a rotary evaporator. The flask was raised from the water bath and the products solidified as the rotating flask cooled under the influence of the vacuum. Then the suspension was cooled for 1 hour on an ice bath. A white solid was collected by vacuum filtration with the protection of argon. The filter cake was washed with a total of 20 ml ice cold 10% methanol/isopropanol followed by 10 ml of room temperature ethyl ether. The damp product is dissolved in 20 ml methanol, then slowly added to 40 ml of ice cold isopropanol on a ice bath. The white solid was filtered, washed with 10 ml of ice cold 10% methanol/isopropanol and 10 ml of room temperature of ethyl ether. The product was briefly dried with a stream of argon followed by drying in vacuum P_2O_5 desiccator overnight. About 2.0 gram of the white PEG-15PA-ONp product was obtained, and the product contains about 9.9 ONp groups per PEG-20K molecule as determined by UV absorbance (•401.5nm = 18,400 in 0.1 N NaOH solution).

About 2.0 grams (1.0 mmole ONp ester) of dried PEG20K-15PA-ONp and 608 mg (1.2 equivalents of ONp ester) of dried GFLG tetrapeptide (TFA salt) were dissolved in 20 ml of anhydrous DMF. About 0.48 ml (2.76 mmoles, 2 equivalents of GFLG) of N,N-diisopropylethylamine (DIPEA) were added to the solution. The reaction mixture was stirred at room temperature for 4 hours. The reaction mixture was concentrated to about 10 ml. To residue was added about 100 ml of cold ethyl ether to precipitate the product. The white solid was filtered off to give about 4 grams of crude product. It was purified on a gel filtration column (2.0 X 80 cm of Sephadex G25, eluted with 0.1 mM triethylamine/acetic acid buffer (pH = 10)) to give 1.75 gram of pure product. ^{1}H-NMR indicate that each copolymer molecule of a PEG20K contains about 9 GFLG tetrapeptide: ^{1}H-NMR (D_2O, 400

MHz), • 7.2 (d, 2.62, ArH of Phe), 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 0.78 (d, 38.3, CH_3 of Leu).

The purified PEG30K-15PA-GFLG product was reacted with PEI400 to form PEG20K-15PA-GFLG-PEI400. About 1.0 gram of PEG20K-15PA-GFLG was reacted with about 20 grams of polyethylenimine of mean molecular weight of about 400 (PEI400) as describe in Example 1. About 1.1 grams of PEG20K-15PA-GFLG-PEI2K was obtained. ^{1}H-NMR indicates that each copolymer molecule contains 2,000 PEI and 9.0 molecules of GFLG linker assuming the average molecular weight of the starting PEG15PA is 20,000. ^{1}H-NMR (D_2O, 400 MHz), 8 7.2 (m, 2.5, ArH of Phe), 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 2.4-3.2 (m, 10, -CH_2CH_2N- of PEI), 0.78 (d, 2.6, CH_3 of Leu).

Example 7 - Synthesis of PEG20K-15PA-GFLG-PEI800 (15 PEI800 grafted PEG20K with GFLG linkers)

Following the procedure of Example 5, PEG20K-15PA reacted with GFLG and polyethylenimine 800 (PEI800) to produce PEG20K-15PA-GFLG-PEI800. ^{1}H-NMR indicates that each copolymer molecule contains 4,400 PEI and 9.0 molecules of GFLG linker assuming the average molecular weight of the starting PEG 15PA is 20,000. ^{1}H-NMR (D_2O, 400 MHz), 8 7.2 (m, 2.5, ArH of Phe), 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 2.4-3.2 (m, 22, -CH_2CH_2N- of PEI), 0.78 (d, 2.6, CH_3 of Leu).

Example 8 - Synthesis of PEG20K-8PA-GFLG-PEI400 (8 PEI400 grafted PEG20K with GFLG linkers)

Following the procedure of Example 5, PEG20K-8PA reacted with GFLG and polyethylenimine 400 (PEI400) to produce PEG20K-8PA-GFLG-PEI400. ^{1}H-NMR indicates that each copolymer molecule contains 1,087 PEI and 3.8 molecules of GFLG linker, assuming the average molecular weight of the starting PEG-8PA is 20,000. ^{1}H-NMR (D_2O, 400 MHz), 8 7.2 (m, 1.1, ArH of Phe), 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 2.4-3.2 (m, 5.6, -CH_2CH_2N- of PEI), 0.78 (d, 1.1, CH3 of Leu).

Example 9 - Synthesis of PEG20K-8PA-GFLG-PEI800 (8 PEI800 grafted PEG20K with GFLG linkers)

Following the procedure of Example 5, PEG20K-8PA reacted with GFLG and polyethylenimine 800 (PEI800) to produce PEG20K-8PA-GFLG- PEI800. ^{1}H-NMR indicates that each copolymer molecule contains 2,207 PEI and 3.8 molecules of GFLG linker assuming the average molecular weight of the starting PEG15PA is 20,000. ^{1}H-NMR (D_2O, 400 MHz), 8 7.2 (m, 1.1, ArH of Phe), 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 2.4-3.2 (m, 11.3, -CH_2CH_2N- of PEI), 0.78 (d, 1.1, CH_3 of Leu).

Example 10 - Synthesis of PEG20K-8PA-GFLG-PEI2K (8 PEI2K grafted PEG 20K with GFLG linkers)

Following the procedure of Example 5, PEG20K-8PA reacted with GFLG and polyethylenimine with mean molecular weight of 1800 (PEI2K) to produce PEG20K-8PA-GFLG-PEI2K. ^{1}H-NMR indicates that each copolymer molecule contains 6,297 PEI and 3.8 molecules of GFLG linker, assuming the average molecular weight of the starting PEG-8PA is 20,000. ^{1}H-NMR (D_2O, 400 MHz), 8 7.2 (m, 1.1, ArH of Phe), 3.4-3.8 (m, 100 (arbitrarily set), -CH_2CH_2O- of PEG), 2.4-3.2 (m, 26, -CH_2CH_2N- of PEI), 0.78 (d, 1.1, CH_3 of Leu).

Example 11 - Transfection of plasmid DNA to cultured cells using copololymers

HT1080 cells were seeded on a 6-well tissue culture plate. The cells were seeded at about 100,000 cells per well in 1.0 ml of HyQ MEM/EBSS medium with 10% FBS. The plate was incubated overnight at 37 °C in a 5% CO_2 incubator. Next, the medium was removed by aspiration and 900 µL of fresh medium was added to each well.

A transfection medium was prepared containing a complex of DNA and a carrier copolymer of the present invention. A solution of the carrier copolymer was created. The concentration of the carrier copolymer was normalized to about 0.6 mg/ml PEI in PBS buffer. Next a volume of the carrier copolymer solution ranging from about 2.0 µL to about 20 µL was added to about 100 µL of serum free media in a sterile tube. The resulting solution was incubated for about 10 minutes at room temperature. The solution was then mixed with about 2.0 µL of 1.0 µg/µL of green fluorescent protein DNA (GFP) or red fluorescent protein DNA (RFP) solution and incubated 20 minute at room temperature to create a DNA/carrier copolymer complex.

The DNA/carrier copolymer complex was added drop wise to the cells in the 6 well plate. As the complex was added to the cells, the plate was gently rocked in all directions to mix the complex with the growth medium. The cells were then incubated for at least 24 hours at 37 °C in a 5% CO_2 incubator. The cells were examined with a fluorescence microscope, or a FACS cell sorter. The transfection medium was removed by aspiration and fresh medium was added to preserve the cells. Table 1 shows the results of the transfection experiments using various carriers of the present invention and controls and plasmid GFP DNA. A plus indicates successful transfection and a minus indicates no transfection.

Example 12 - Transfection of oligonucleotide to cultured cells using copolymers

About 2,500 cells per well were seeded on a 96 well tissue culture plate. The cells were incubated overnight at 37°C in a 5% CO_2 incubator. Then the old medium was removed by aspiration and 50 µL of fresh medium with 10% FBS was added.

A transfection medium was prepared containing the a complex of an oligonucleotide and the carrier copolymer of the present invention. A solution of the carrier copolymer was created. The concentration of the carrier copolymer was about 0.6 mg/ml PEI in PBS buffer. Next, a volume ranging from about 2.0 μL to about 20 μL of the carrier copolymer solution was added to a volume of serum free media to make a total solution volume of 50 μL in a sterile tube. The resulting solution was incubated at room temperature for about 10 minutes.

The solution was then mixed with about 2.0 μL of 0.1mM oligonucleotide solution (22-mer, ~0.7 mg/ml). The oligonucleotide contained was a 22-mer phosphodiester oligonucloetide with a 3' inversion and 5' fluorescence labeling. The resulting transfection medium was then incubated for 20 minutes at room temperature. The transfection medium was added to the wells in the 96 well plate. The cells were then incubated at 37 °C in a 5% CO_2 incubator for about 6 hours. Next, the transfection medium was removed by aspiration and the cells were washed twice with about 100 μL of sterile PBS.

After washing, about 100 μL of fresh medium was added to the wells and the cells were viewed under fluorescence microscope. Fluorescence indicated that the cells were successfully transfected with the oligonucleotide. Table 1 shows the results of the transfection experiments using different carriers of the present invention and controls and the oligonucleotide. A plus indicates successful transfection and a minus indicates no transfection.

Table 1. Summary of the copolymer structures and their transfection activities on plasmid DNA and oligonucleotides. The chemical structures were carefully characterized using ^{1}H-NMR on a Varian 400 MHz machine. The DNA and oligonucleotide binding stability was tested by gel shift assay as described. Gene transfection was tested using plasmid DNA containing GFP reporting gene. Oligonucleotide transfection was tested using a 22-mer phosphodiester oligonucleotide with 3'-end inversion and 5'-end fluorescence labeling.

Copolymer	$N_{(GFLG)}$	$W_{(PEI)}$	Mw	DNA/oligo Binding	Transfection psDNA	Transfection Oligo
PEG-15PA-PEI400	0.0	2,344 (10%)	23,444	+	-	-
PEG-15PA-PEI800	0.0	8,400 (30%)	28,400	+	+	+
PEG-8PA-PEI800	0.0	2,607 (11.5%)	22,607	+	-	+
PEG-10PA-PEI1200	0.0	4,963 (20.0%)	24,936	+	+	+
PEG-8PA-PEI2K	0.0	7,490 (27%)	27,490	+	+	+
PEG-15PA-GFLG-PEI400	9.0	2,000 (7.8%	25,400	-	-	-
PEG-15PA-GFLG-PEI800	9.0	4,400 (15.8%)	27,900	+	+	+
PEG-8PA-GFLG-PEI400	3.8	1,087 (4.8%)	22,577	+	-	+
PEG-8PA-GFLG-PEI800	3.8	2.207 (9.3%)	23,697	+	+	+
PEG-8PA-GFLG-PEI2K	3.8	4,975 (18,8%)	26,465	+	+	+
Controls:						
PEI-25K (from Sigma)			25,000	+	+	+
PEI-2K (from Polysciences)			1,800	+	-	+
PEI-400 (from Sigma)			400	-	-	-

SUMMARY

In summary, the invention presents a novel class of polycation grafted polymeric carrier molecules. The novel polycation grafted copolymers exhibit substantial water solubility and a low level of toxicity. Certain embodiments of the invention uses PEG as a backbone polymer to which PEI fragments or other polycationic polymer fragments are attached. PEG is linear polymer with many useful properties, such as good solubility and good excretion kinetics. Additionally, PEG is biocompatibility because of its minimal toxicity, immunogenicity and antigenicity. These features have made PEG the most extensively studied drug carrier in pharmaceutical research which had been approved by FDA for internal administration. By conjugating a polycationic polymer to a biocompatible polymers such as PEG, the polycationic polymer can be rendered more soluble and less toxic. Additionally small polycationic polymer fragments are much less toxic than large molecular weight cationic polymers and could be easily cleared out from the body. Thus, by conjugating the small cationic polymers to a biocompatible backbone polymer carrier copolymers can be created that allow for delivery of therapeutics such as polyanionic macromolecules to cells.

The carrier polymers of the present invention also provide enhanced stability of the complexed DNA/carrier copolymer stability. The carrier polymers of the present invention have also been to shown to have enhanced transfection activity compared to other DNA carrier polymers. Unlike the unmodified polycations which form aggregation precipitates when complexed with nucleic acids, the copolymers of this invention bind nucleic acids through ionic interaction to form a coreshell like micelle structure. This structure is stable and soluble in biological conditions due to the neutral hydrophilic shell formed by the biocompatible backbone polymer. The complex is stable in biological buffers, even with the presence of serum. As a result, the transfection activity is much higher than the unmodified polycation carriers, such as PEI, PLL or chitosan.

The carriers of the present invention can be used to deliver drugs and other therapeutic agents to specifically targeted cells or tissues. The copolymer carrier can be used for the controlled release and targeted delivery of nucleic acids to a cell. Moreover, the efficiency of a drug efficiency can be increased by targeting specific cells or organs, therefore reducing accumulation of the drug in healthy tissues and minimizing its toxicity. Such specific targeting allows higher doses of a therapeutic to be administered, if needed, without undesired effects on non targeted cells.

CLAIMS:

1. A carrier for transporting a polyanionic macromolecule across a biological barrier of a cell comprising:

a biocompatible hydrophilic backbone polymer; and

two or more polycationic polymers covalently linked to the biocompatible hydrophilic backbone polymer by a linker.

2. The carrier of claim 1, wherein the biocompatible hydrophilic backbone is selected from the group consisting of polyethylene glycol (PEG), poly (N-(2-hydroxylpropyl)methacrylamide), and copolymers thereof.

3. The carrier of claim 2, wherein the polycationic polymers are polyethylenimine (PEI).

4. The carrier of claim 1, wherein the polycationic polymers are selected from the group consisting of polyalkylamine (PAM), polyethylenimine (PEI), polylysine (PL), a polypeptide, chitosan, a polysaccharide, and copolymers thereof.

5. The carrier of claim 1, further comprising at least one targeting moiety connected to the biocompatible hydrophilic backbone or to one of the two or more polycationic polymers.

6. The carrier of claim 5, wherein the targeting moiety is selected from the group consisting of a ligand, an antigen, a hapten, biotin, lectin, galactose, galactosamine, a protein, a histone, a polypeptide, a lipid, a carbohydrate, a vitamin, and a combination thereof.

7. The carrier of claim 1, further comprising at least one lysis agent connected to the biocompatible hydrophilic backbone or to one of the two or more polycationic polymers.

8. The carrier of claim 7, wherein the at least one lysis agent is selected from the group consisting of a viral peptide, a bacterial toxin, a lytic peptide, aleveolysin, bifermentolysin, boutulinolysin, capriciolysin, cereolysin O, chauveolysin, histolyticolysin O, pneumolysin, sealigerolysin, septicolysin O, sordellilysin, streptoslysin O, tenaolysin or thuringolysin O, and active fragments thereof.

9. The carrier of claim 1, wherein the linker has a length from about 2 to about 100 atoms.

10. The carrier of claim 9, wherein the linker is selected from the group consisting of a hydrocarbon chain, a PEG fragment, a polypeptide, a linear polymer containing an ester bond, a linear polymer containing an amide bond, a linear polymer containing a disulfide bond, a linear polymer containing a hydrozone bond, and a linear polymer containing an oxime bond.

11. The carrier of claim 9, wherein the length of the linker is within the range from about 3 atoms to about 30 atoms.

12. The carrier of claim 1, wherein the biocompatible hydrophilic backbone has a molecular weight in the range from about 1,000 to about 1,000,000 and the polycationic polymers have a molecular weight in the range from about 100 to about 100,000.

13. The carrier of claim 12, wherein the molecular weight of the biocompatible hydrophilic backbone is in the range from about 5,000 to about 100,000.

14. The carrier of claim 12, wherein the molecular weight of the biocompatible hydrophilic backbone is in the range from about 20,000 to about 40,000.

15. The carrier of claim 12, wherein the molecular weight of the polycationic polymers is in the range from about 200 to about 10,000.

16. The carrier of claim 12, wherein the molecular weight of the polycationic polymers is in the range from about 400 to about 2,000.

17. The carrier of claim 1, wherein the biocompatible hydrophilic backbone is polyethylene glycol and the polycationic polymers is polyethylenimine.

18. The carrier of claim 17, wherein from about 4 to about 100 polycationic polymers are covalently linked to the biocompatible hydrophilic backbone polymer by a linker.

19. The carrier of claim 17, wherein from about 8 to about 15 polycationic polymers are covalently linked to the biocompatible hydrophilic backbone polymer by a linker.

20. The carrier of claim 17, wherein the molecular weight of the biocompatible hydrophilic backbone is in the range from about 20,000 to about 40,000.

21. The carrier of claim 17, wherein the molecular weight of polycationic polymers is in the range from about 400 to about 2,000.

22. The carrier of claim 17, wherein the linker is selected from the group consisting of a hydrocarbon chain, a PEG fragment, a polypeptide, a linear polymer containing an ester bond, a linear polymer containing an amide bond, a linear polymer containing a disulfide bond, a linear polymer containing a hydrozone bond, and a linear polymer containing an oxime bond.

23. The carrier of claim 17, further comprising at least one targeting moiety connected to the biocompatible hydrophilic backbone or to one of the two or more polycationic polymers.

24. The carrier of claim 23, wherein the targeting moiety is selected from the group consisting of a ligand, an antigen, a hapten, biotin, lectin, galactose, galactosamine, a protein, a histone, a polypeptide, a lipid, a carbohydrate, a vitamin, and a combination thereof.

25. The carrier of claim 17, further comprising at least one lysis agent connected to the biocompatible hydrophilic backbone or to one of the two or more polycationic polymers.

26. The carrier of claim 25, wherein the at least one lysis agent is selected from the group consisting of a viral peptide, a bacterial toxin, a lytic peptide, aleveolysin, bifermentolysin, boutulinolysin, capriciolysin, cereolysin O, chauveolysin, histolyticolysin O, pneumolysin, sealigerolysin, septicolysin O, sordellilysin, streptoslysin O, tenaolysin or thuringolysin O, and active fragments thereof.

27. The carrier of claim 17, wherein the linker is a biodegradable peptide.

28. The carrier of claim 1, wherein from about 4 to about 100 polycationic polymers are covalently linked to the biocompatible hydrophilic backbone polymer by a linker.

29. The carrier of claim 1, wherein from about 8 to about 15 polycationic polymers are covalently linked to the biocompatible hydrophilic backbone polymer by a linker.

30. The carrier of claim 25, wherein the biodegradable peptide is selected from the group consisting of GlyPhePheGly and GlyPheLeuGly.

31. A complex for transporting a polyanionic macromolecule across a biological barrier of a cell comprising:

a carrier molecule for delivering the polyanionic macromolecule to the cell, the carrier molecule comprising a biocompatible hydrophilic backbone polymer and two or more polycationic polymers covalently linked to the biocompatible hydrophilic backbone polymer by a linker; and

a polyanionic macromolecule complexed with the carrier molecule.

32. The complex of claim 31, wherein the polyanionic macromolecule is a nucleic acid.

33. The complex of claim 32, wherein the polycationic polymers are PEI.

34. The complex of claim 33, wherein the biocompatible hydrophilic backbone polymer is PEG.

35. The complex of claim 33, wherein the biocompatible hydrophilic backbone polymer is HPMA.

36. The complex of claim 32, wherein the nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, synthetic DNA, and RNA.

37. The complex of claim 32, wherein the nucleic acid is selected from the group consisting of an antisense oligonucleotide, ribozyme, DNAzyme, chimeric RNA/DNA oligonucleotide, phosphorothioate oligonucleotide, 2'-O-methyl oligonucleotide, DNA-PNA conjugate, DNA-morpholino-DNA conjugate, and a combination thereof.

38. The complex of claim 31, wherein the biocompatible hydrophilic backbone has a molecular weight in the range from about 1,000 to about 1,000,000 and the polycationic polymers have a molecular weight in the range from about 100 to about 100,000.

39. The complex of claim 38, wherein the molecular weight of the biocompatible hydrophilic backbone is in the range from about 20,000 to about 40,000.

40. The complex of claim 39, wherein the molecular weight of the polycationic polymers is in the range from about 400 to about 2,000.

41. The complex of claim 31, wherein the linker is selected from the group consisting of a hydrocarbon chain, a PEG fragment, a polypeptide, a linear polymer containing an ester bond, a linear polymer containing an amide bond, a linear polymer containing a disulfide bond, a linear polymer containing a hydrozone bond, and a linear polymer containing an oxime bond.

42. The complex of claim 31, wherein the biocompatible hydrophilic backbone is selected from the group consisting of polyethylene glycol (PEG), poly (N-(2-hydroxylpropyl)methacrylamide), and copolymers thereof.

43. The complex of claim 42, wherein the polycationic polymers are selected from the group consisting of polyalkylamine (PAM), polyethylenimine (PEI), polylysine (PL), a polypeptide, chitosan, a polysaccharide, and copolymers thereof.

44. The complex of claim 31, further comprising at least one targeting moiety connected to the biocompatible hydrophilic backbone or to one of the two or more polycationic polymers, the at least one targeting moiety selected from the group consisting of a ligand, an antigen, a hapten, biotin, lectin, galactose, galactosamine, a protein, a histone, a polypeptide, a lipid, a carbohydrate, and a combination thereof.

45. The complex of claim 31, further comprising at least one lysis agent connected to the biocompatible hydrophilic backbone or to one of the two or more polycationic polymers, the at least one lysis agent selected from the group consisting of a viral peptide, a bacterial toxin, a lytic peptide, aleveolysin, bifermentolysin, boutulinolysin, capriciolysin, cereolysin O, chauveolysin, histolyticolysin O, pneumolysin, sealigerolysin, septicolysin O, sordellilysin, streptoslysin O, tenaolysin or thuringolysin O, and active fragments thereof.

46. The complex of claim 31, wherein from about 4 to about 100 polycationic polymers are covalently linked to the biocompatible hydrophilic backbone polymer by a linker.

47. The complex of claim 31, wherein from about 8 to about 15 polycationic polymers are covalently linked to the biocompatible hydrophilic backbone polymer by a linker.

48. A method of transporting a polyanionic macromolecule across a biological barrier of a cell comprising:

complexing the polyanionic macromolecule to a carrier molecule to create a complex, the carrier molecule comprising a biocompatible hydrophilic backbone polymer and two or more polycationic polymer covalently linked to the biocompatible hydrophilic backbone polymer by a linker; and

contacting the cell with the complex.

49. The method of claim 48, wherein the biocompatible hydrophilic backbone is selected from the group consisting of polyethylene glycol (PEG), poly (N-(2-hydroxylpropyl)methacrylamide), and copolymers thereof.

50. The method of claim 49, wherein the polycationic polymers are selected from the group consisting of polyalkylamine (PAM), polyethylenimine (PEI), polylysine (PL), a polypeptide, chitosan, a polysaccharide, and copolymers thereof.

51. The method of claim 48, further comprising at least one targeting moiety connected to the biocompatible hydrophilic backbone or to one of the two or more polycationic polymers, the targeting moiety selected from the group consisting of a ligand, an antigen, a hapten, biotin, lectin, galactose, galactosamine, a protein, a histone, a polypeptide, a lipid, a carbohydrate, and a combination thereof.

52. The method of claim 48, further comprising at least one lysis agent connected to the biocompatible hydrophilic backbone or to one of the two or more polycationic polymers, the at least one lysis agent selected from the group consisting of a viral peptide, a bacterial toxin, a lytic peptide, aleveolysin, bifermentolysin, boutulinolysin, capriciolysin, cereolysin O, chauveolysin, histolyticolysin O, pneumolysin, sealigerolysin, septicolysin O, sordellilysin, streptoslysin O, tenaolysin or thuringolysin O, and active fragments thereof.

53. The method of claim 48, wherein the linker has a length from about 2 to about 100 atoms.

54. The method of claim 53, wherein the linker is selected from the group consisting of a hydrocarbon chain, a PEG fragment, a polypeptide, a linear polymer containing an ester bond, a linear polymer containing an amide bond, a linear polymer containing a disulfide bond, a linear polymer containing a hydrozone bond, and a linear polymer containing an oxime bond.

55. The method of claim 53, wherein the linker is a biodegradable peptide.

56. The method of claim 55, wherein the biodegradable peptide is selected from the group consisting of GlyPhePheGly and GlyPheLeuGly.

57. The method of claim 48, wherein the biocompatible hydrophilic backbone has a molecular weight in the range from about 1,000 to about 1,000,000 and the polycationic polymers have a molecular weight in the range from about 100 to about 100,000.

58. The method of claim 57, wherein the molecular weight of the biocompatible hydrophilic backbone is in the range from about 20,000 to about 40,000.

59. The method of claim 57, wherein the molecular weight of the polycationic polymers is in the range from about 400 to about 2,000.

60. The method of claim 57, wherein the biocompatible hydrophilic backbone is polyethylene glycol and the polycationic polymers are polyethylenimine.

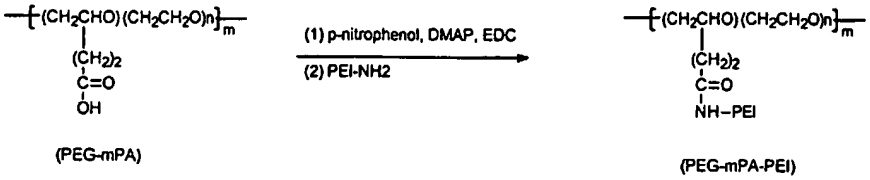
61. The method of claim 60, wherein the molecular weight of the biocompatible hydrophilic backbone is in the range from about 20,000 to about 40,000.

62. The method of claim 60, wherein the molecular weight of the polycationic polymers is in the range from about 400 to about 2,000.

63. The method of claim 48, wherein from about 4 to about 100 polycationic polymers are covalently linked to the biocompatible hydrophilic backbone polymer by a linker.

64. The method of claim 48, wherein from about 8 to about 15 polycationic polymers are covalently linked to the biocompatible hydrophilic backbone polymer by a linker.

A



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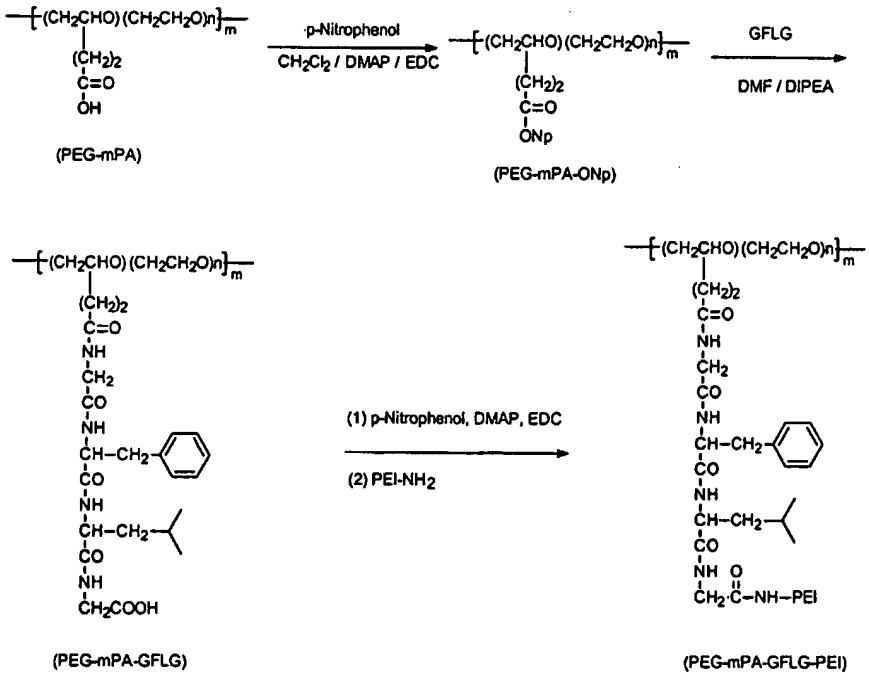

DMAP = 4-dimethylaminopyridine

EDC = 1-[3-dimethylaminopropyl]-3-ethylcarbodiimide hydrochloride)

PEI-NH_2 = $H_2NCH_2CH_2-[NHCH_2CH_2]_x-NHCH_2CH_2NH_2$ (Linear PEI) or $-[NHCH_2CH_2]_x-[NCH_2CH_2(CH_2CH_2NH_2)]_y-$ (Branched PEI)

Figure 1

SEQUENCE LISTING

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<120> POLYCATIONIC WATER SOLUBLE COPOLYMER AND METHOD FOR TRANSFERRING POLYANIONIC MACROMOLECULES ACROSS BIOLOGICAL BARRIERS

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 June 2003 (12.06.2003)

PCT

(10) International Publication Number
WO 03/047518 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/US02/38223

(22) International Filing Date:
27 November 2002 (27.11.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/999,252 30 November 2001 (30.11.2001) US

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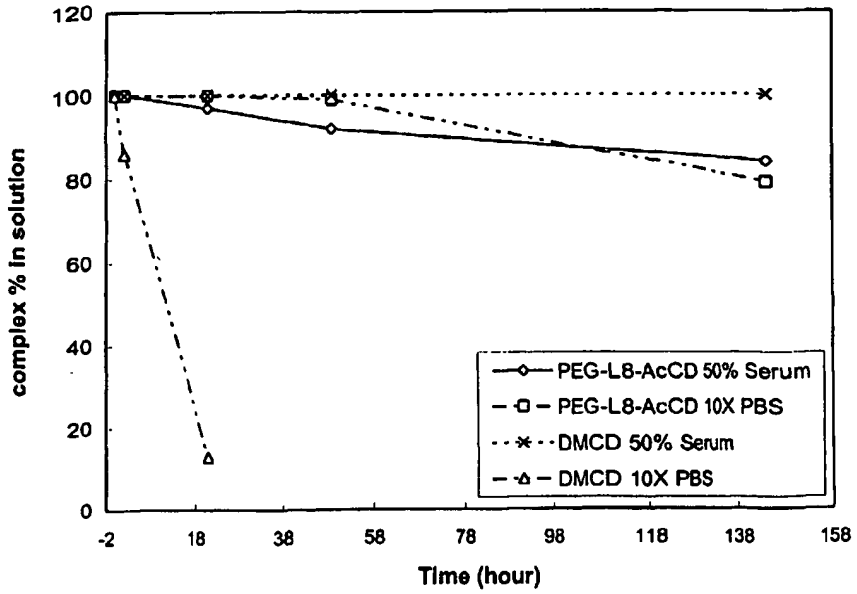
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK,

[Continued on next page]

(54) Title: CYCLODEXTRIN GRAFTED BIOCOMPATIBLE AMPHIPHILIC POLYMER AND METHODS OF PREPARATION AND USE THEREOF



(57) Abstract: Amphiphilic biocompatible cyclodextrin grafted polymers comprising a hydrophobically modified cyclodextrin moiety, a linear linker and a biocompatible hydrophilic polymer backbone, wherein said cyclodextrin moiety is grafted to said biocompatible hydrophilic polymer backbone by said linker are disclosed. The cyclodextrin-grafted biocompatible polymers of this invention may be used as bioactive agent carriers. Methods of making and using such cyclodextrin-grafted biocompatible polymers are disclosed.

WO 03/047518 A2

TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

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CYCLODEXTRIN GRAFTED BIOCOMPATIBLE AMPHIPHILIC POLYMER AND METHODS OF PREPARATION AND USE THEREOF

BACKGROUND OF THE INVENTION

This invention relates to novel polymeric bioactive agent carriers. More particularly, the invention relates to cyclodextrin-grafted biocompatible polymers used as bioactive agent carriers and methods of making thereof.

Many biologically active molecules such as anti-viral agents, anti-cancer agents, peptides/proteins and DNA, effective for a variety of therapeutic applications, have become commercially available through advances in recombinant DNA and other technologies. However, an ideal carrier for drugs and active agents is always needed to facilitate their solubility, delivery and effectiveness.

Cyclodextrins (CDs) are cyclic oligosaccharides, usually consisting of six to eight glucose units, which have a truncated cone shape with the wide open side being formed by secondary hydroxyl groups (2-OHs and 3-OHs) and the narrower side by primary hydroxyl groups(6-OHs). Cyclodextrins provide for unique micro-heterogenous environments since the exterior of the molecule is hydrophilic while the cavity is hydrophobic due to the relatively high electron density. The inclusion properties of cyclodextrins, namely, complex-formation between a guest molecule and a cyclodextrin molecule, have been extensively investigated. The complexes, which are formed in the solid state and in solution, consist of guest molecules which are held in the cavity of the host cyclodextrin and are stabilized by Van der Waals forces, and, to a lesser extent, by dipole-dipole interactions. Inclusion complexes in aqueous solutions are thought to be further stabilized by hydrophobic interactions, i.e., by the tendency of solvent water to push hydrophobic solutes of suitable size and shape into the essentially hydrophobic cavity, in order to attain the "most probable structure" of the solvent and obtain minimal energy in the overall system.

Practical use of natural cyclodextrins (α-, β-, and γ-CDs) as drug carriers is restricted by their low aqueous solubility. Safety is another major concern of cyclodextrins being used as drug carriers due to the toxicity of CD. Modification of

the parent cyclodextrin to improve safety while maintaining the ability to form inclusion complexes with various substrates has been the goal of numerous research groups. Some groups have also focused on improving interaction between the pharmaceuticals and the cyclodextrins while others have attempted to prepare materials that can be chemically defined more precisely.

The two most promising cyclodextrin derivatives which are suitable for parenteral administration are hydoxylpropyl β-cyclodextrin (HPβCD or HPCD) and sulfobutylether- β-cyclodextrin (SBEβCD or SBE-CD). HPβCD has generally been found to be safe when administered parenterally in animals and humans [Pitha et al, *J Pharm Sci*, 84 (8), 927-32 (1995)]. Minor reversible histological changes have been observed in high dose animal studies (100-400 mg/kg) and more significant hematological changes were observed in these high dose studies suggesting red blood cell damage had occurred. No adverse effects were observed in human studies. SBEβCD has also been found to be safe when administered parenterally in mice [Rajewski et al, *J Pharm Sci*, 84 (8), 927-32 (1995)]. However, like most of the modified cyclodextrins, the binding constant between drugs and HPβCDs is usually less than those with the parent or unmodified cyclodextrin. Due to steric hindrance of the host molecule, the higher the degree of hydroxylpropyl substitution the poorer the drug binding.

Hydrophobic modifications of cyclodextrins have also been prepared in attempts to improve the formulations of some CD inclusionable drugs. It was found that partial methylation of the hydroxyl groups at the 2- and 6-position of β-cyclodextrin (DM-βCD or DMCD) generally leads to stronger drug binding due to increased hydrophobic interactions. Although the methylated cyclodextrins are highly water soluble, they also have greater toxicity. The toxicity of DMβCD was reduced significantly by modifying the free 3-hydroxyl groups with acetyl groups. This indicates that water-soluble cyclodextrin derivatives with superior bioadaptability and inclusion ability can be prepared by carefully selecting the substitution groups. Controlling the degree of substitution is also important in balancing water solubility and complexing capability. When the substitution groups are more hydrophobic than methyl groups, such as an ethyl group, an acetyl group, etc., the whole cyclodextrin

derivative becomes practically water insoluble. These compounds have been shown to have potential application as sustained release carriers for water-soluble drugs. Among the alkylated cyclodextrins, heptakis(2,6-di-O-ethyl)-β-cyclodextrin and heptakis(2,3,6-tri-ethy)-β-cyclodextrin were the first slow-release carriers to be used in conjunction with water soluble diltiazem, isosorbide dinitrate, and the peptide buserelin acetate.

On the other hand, the peracylated cyclodextrins with medium alkyl chain lengths (C_4-C_5) are particularly useful as novel hydrophobic carriers due to their multifunctional and bioadaptable properties. They have broad applicability for various routes of administration: for example, the bioadhesive properties of heptakis(2,3,6-tri-O-butanoyl-β-cyclodextrin (C_4) can be used in oral and transmucosal formulations, while the film-forming properties of heptakis (2,3,6-tri-O-valeryl)-b-cyclodextrin (C_5) are useful in transdermal preparations. In oral applications, the release of molsidomine, a water-soluble and short-half life drug, was markedly retarded by complexation with peracylated-β-cyclodextrins in decreasing order of their solubility, particularly by those having carbon chains longer than the butylated derivatives. When the complexes were administered orally to beagle dogs, heptakis(2,3,6-tri-O-butanoyl)-β-cyclodextrin suppressed the peak plasma level of molsidomine and maintained a sufficient drug level for a long period, while use of other derivatives having shorter or longer chains than heptakis(2,3,6-tri-O-butanoyl)- β-cyclodextrin proved to be insufficient. This indicates that heptakis(2,3,6-tri-O-butanoyl)- β-cyclodextrin may be a useful carrier for orally administered water-soluble drugs, especially for drugs which are metabolized in the GI tract. The superior and sustained effect exhibited with the heptakis (2,3,6-tri-O-butanoyl)-β-cyclodextrin may be a result of both increased hydrophobicity and mucoadhesive properties. Because of its hydrophobicity, heptakis(2,3,6-tri-O-butanoyl)-β-cyclodextrin, as well as other hydrophobic cyclodextrin derivatives, can only be used in solid or oily formulations. On the other hand, like natural β-cyclodextrin, their membrane toxicity, which causes tissue irritation and hemolysis in a concentration-dependent manner is another limitation of their pharmaceutical application. For example, the concentration of DM-β-CD that induces 50% hemolysis of human erythrocytes is lower than that of so

called bioadaptable CD derivatives such as 2-hydroxypropyl-β-CD, sulfobutyl ether of β-CD, and maltosyl-β-CD. The hemolytic activity of cyclodextrins is associated with the extraction of membrane components, mainly through inclusion action with cholesterol. However, this drawback can be overcome by further structural modification of alkylated CDs, for example, heptakis(2,6-di-O-methyl-3-O-acetyl)-β-CD (DMA-β-CD) was found to have much weaker hemolytic activity while keeping a similar inclusion ability to that of DM-β-CD [Hirayama et al, *J Pharm Sci*, 88 (10), 970-5 (1999)]. Since cyclodextrins are poorly adsorbed from the GI tract following oral administration, the oral administration of cyclodextrins raises minimal safety concerns that may result from the systemic absorption of the cyclodextrins themselves. However, cyclodextrins may cause secondary systemic effects through increased GI elimination of certain nutrients and bile acids. This effect is most notable for ύ-cyclodextrin assisted fecal elimination of bile acids. The increased elimination, however, was only observed at very high oral doses of cyclodextrin (up to 20% of diet). The secondary effects of the increased bile acid elimination are increased conversion of serum cholesterol to the bile acids with subsequent lowering of plasma cholesterol levels.

For years, various kinds of cyclodextrins have been prepared to improve the physicochemical properties and inclusion capabilities of parent cyclodextrins, and some of the pharmaceutical products containing cyclodextrins have been approved. Because large amounts of cyclodextrins are necessary to alter the solubility properties of the drugs being carried, the toxicity of the cyclodextrin needs to be very low in order to safely delivery the necessary dose of a drug. Therefore either reducing the total dose or reducing the intrinsic toxicity of cyclodextrins can widen the pharmaceutical applications of cyclodextrins.

In view of the foregoing, it will be appreciated that providing improved cyclodextrin containing bioactive agent carriers and a method of using them would be a significant advancement in the art.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a new class of amphiphilic cyclodextrin containing polymers wherein multiple hydrophobic cyclodextrin or derivitized cyclodextrin moieties are conjugated with or grafted to a biocompatible hydrophilic polymeric backbone, through appropriate biodegradable or non-biodegradable linkers. Optionally, one or more or a mixture of targeting moieties (TM) may also be covalently bound to the polymeric backbone. The CD-grafted polymers of the present invention can be synthesized by coupling two to thirty CDs or derivatives thereof to a hydrophilic polymer, i.e. a polyethylene glycol (PEG) or poly N-(2-hydroxylpropyl)methacrylamide) (HPMA), via a proper linker. If desired, as described above, one or more targeting moieties(TM) may optionally be covalently attached to the polymer backbone. The purpose of using the targeting moiety is to target particular cells for drug delivery. The synthesized carrier, namely a hydrophobic CD-grafted hydrophilic polymer, results in better solubility and reduced cytotoxicity of the drug/carrier complex.

DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIG. 1 is a graphic illustration showing the stability of Paclitaxel/CD complexes in 50% serum or 10xPBS dilutions.

FIG.2 depicts a reaction scheme for synthesis of PEG-SS-AcCD

FIG.3 depicts a reaction scheme for synthesis of PEG-SS-DECD

FIG.4 depicts a reaction scheme for synthesis of PEG-GFLG-DECD

FIG.5 depicts a reaction scheme for synthesis of PEG-C3-AcCD, PEG-C3-DECD and PEG-C3-BnCD.

FIG.6 depicts a reaction scheme for synthesis of PEG-L8-AcCD, PEG-L8-DECD.

DETAILED DESCRIPTION OF THE INVENTION

Before the present composition and method for drug delivery are disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the

terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

"Active agents" refers to those agents that can function as guest molecules of the instant invention. Active agents include chemicals and other substances which can form an inclusion complex with a cyclodextrin or derivatized cyclodextrin grafted polymer and are inhibitory, antimetabolic or preventive toward any disease (i.e. cancer, syphilis, gonorrhea, influenza and heart disease) or inhibitory or toxic toward any disease causing agent. Active agents include numerous drugs such as anticancer drugs, antineoplastic drugs, antifungal drugs, antibacterial drugs, antiviral drugs, cardiac drugs, neurological drugs, and drugs of abuse; alkaloids (i.e. camptothecins), antibiotics, bioactive peptides, steroids, steroid hormones, polypeptide hormones, interferons, interleukins, narcotics, nucleic acids including antisense oligonucleotides, pesticides and prostaglandins. Active agents also include aflatoxins, ricins, bungarotoxins, irinotecan, ganciclovir, furosemide, indomethacin, chlorpromazine, methotrexate, cevine derivatives and analogs including cevadines, desatrines, veratridine. It also includes various flavone derivatives and analogs including dihydroxyflavones (chrysins), trihydroxyflavones (apigenins), pentahydroxyflavones (morins), hexahydroxyflavones (myricetins), flavyliums, quercetins, fisetins; various antibiotics including penicillin derivatives (i.e. ampicillin), anthracyclines (i.e. doxorubicin, daunorubicin), teramycins, tetracyclines, chlorotetracyclines, clomocyclines, butoconazole, ellipticines, guamecyclines, macrolides (i.e. amphotericins), filipins, fungichromins, nystatins; various purine and pyrimidine derivatives and analogs including 5'-fluorouracil, 5'-fluoro-2'-deoxyuridine, and allopurinol; various photosensitizer substances, especially those used for singlet and triplet oxygen formation useful for photodynamic, phthalocyanine, porphyrins and their derivatives and analogs; various steroid derivatives and analogs including cholesterols, digoxigenins; various coumarin derivatives and analogs including

dihydroxycoumarins (esculetins), dicumarols; chrysarobins, chrysophanic acids, emodins, secalonic acids; various dopas, derivatives and analogs including dopas, dopamines, epinephrines, and norepinephrines (arterenols).

"Parenteral" shall mean intramuscular, intraperitoneal, intra-abdominal, subcutaneous, and, to the extent feasible, intravenous and intraarterial.

"Biocompatible" means that the substance is nonimmunogenic, nonallergenic and will cause minimum undesired physiological reaction. They may be degraded biologically and they are "biologically neutral" in that they lack specific binding properties or biorecognition properties.

"Linkers" or "linkages" are defined as types of specific chemical moieties or groups used within the chemical substances that covalently couple the cyclodextrin moiety to the polymer backbone and may be either biodegradable or non-biodegradable. Suitable linkers are more specifically defined below.

"Drug" shall mean any organic or inorganic compound or substance having bioactivity and adapted or used for a therapeutic purpose. Proteins, hormones, anti-cancer agents, oligonucleotides, DNA, RNA and gene therapies are included under the broader definition of drug.

"Peptide," "polypeptide," "oligopeptide" and "protein" shall be used interchangeably when referring to peptide or protein drugs and shall not be limited as to any particular molecular weight, peptide sequence or length, field of bioactivity or therapeutic use unless specifically stated.

"Targeting moiety" refers to those moieties that bind to a specific biological substance or site. The biological substance or site is considered the "target" of the targeting moiety that binds to it. Examples of suitable targeting moieties are described below. Examples of suitable targeting moieties includes antigens, haptens, biotin, biotin derivatives, lectins, galactosamine and fucosylamine moieties, receptors, substrates, coenzymes, cofactors, proteins, histones, hormones, vitamins, steroids, prostaglandins, synthetic or natural polypeptides, carbohydrates, lipids, antibiotics, drugs, digoxins, pesticides, narcotics, neuro-transmitters, and various nucleic acids.

A "nucleic acid" is defined as any nucleic acid sequence from any source. The nucleic acid includes all types of RNA, DNA, and oligonucleotides including

probes and primers used in polymerase chain reaction (PCR) or DNA sequencing, antisense oligonucleotides and phosphorthioate oligonucleotides. Also included are synthetic nucleic acid polymers, such as methylphosphonate oligonucleotides, phosphotriester oligonucleotides, mopholino-DNA and peptide nucleic acids (PNA) including PNA clamps, DNA and/or RNA fragments, and derivatives from any tissues, cells, nuclei, chromosomes, cytoplasm, mitochondria, ribosomes, and other cellular sources.

A "cyclodextrin (CD)", is a cyclic oligosaccharide composed of glucose monomers coupled together to form a conical, hollow molecule with a hydrophobic interior or cavity. The cyclodextrins of the instant invention can be any suitable cyclodextrin, including alpha-, beta-, and gamma-cyclodextrins, and their combinations, analogs, isomers, and derivatives. Cyclodextrins can be either natural or modified with hydrophobic groups as will be described in greater detail below.

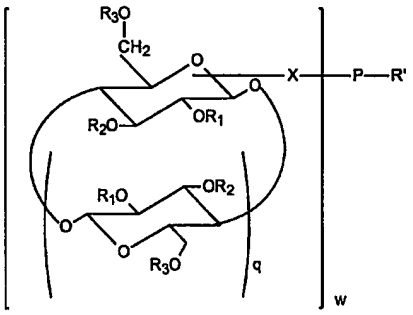
In describing this invention, references to a cyclodextrin "complex", means a noncovalent inclusion complex. An inclusion complex is defined herein as a cyclodextrin or derivatized cyclodextrin functioning as a "host" molecule, combined with one or more "guest" molecules that are contained or bound, wholly or partially, within the hydrophobic cavity of the cyclodextrin or its derivative. Most preferred CDs are derivatives such as carboxymethyl CD, glucosyl CD, maltosyl CD, hydroxypropyl cyclodextrins (HPCD), 2-hydroxypropyl cyclodextrins, 2,3-dihydroxypropyl cyclodextrins (DHPCD), sulfobutylether CD, acylated, ethylated and methylated cyclodextrins. Also preferred are oxidized cyclodextrins that provide aldehydes and any oxidized forms of any derivatives that provide aldehydes. Also included are altered forms, such as crown ether-like compounds and higher homologues of cyclodextrins.

"Controlled release" is defined as the release of a captured guest molecule/drug from the CD polymer carrier only by cleavage of certain linkages that were used to synthesize the carrier.

This invention relates to novel CD-grafted biocompatible amphiphilic polymers and the methods of preparation thereof for use as bioactive agent carriers. The invention, in one of its most general definitions, concerns a complex between a

bioactive agent and at least one CD- grafted polymeric conjugate comprising a biocompatible hydrophilic polymer backbone such as PEG and HPMA, poly-L-Lysine (PLL) and polyethylenimine (PEI) which is grafted with at least one, and preferably a multiplicity, of hydrophobically modified CDs. Optionally a targeting moiety (TM) may be covalently linked to the polymeric carrier.

The preferred cyclodextrin containing polymers may be defined by a cyclodextrin containing -polymer wherein cyclodextrin or derivatized cyclodextrin moieties are connected to a biocompatible hydrophilic polymer backbone by a single spacer arm to the 2, 3, or 6-position of the cyclodextrin which can be represented by Formula 1 as follows:



(1) P is a biocompatible hydrophilic polymer backbone having a molecular weight range from 2,000 to 1,000,000 Daltons, preferably 5,000 to 70,000 Daltons, and most preferably 20,000 to 40,000 Daltons. Preferably the biocompatible polymer backbone is a hydrophilic polymer selected from the group consisting of polyethylene glycol (PEG), N-(2-hydroxypropyl)methacrylamide polymer (HPMA), polyethylenimine (PEI) and polylysine (pLL) which are appropriately endcapped as is known in the prior art and which also may be substituted with substituents that do not adversely affect the functionality of the polymer for its intended purpose. Preferably biocompatible polymer backbone is a polyethylene glycol (PEG) polymer. When the cyclodextrin is attached at the 2, 3 or 6 position of the cyclodextrin the corresponding R_1O- ,R_2O- or R_3O- group will be replaced and the 2-, 3- or 6- carbon of the glucopyranose will be covalently attached to linker X;

(2) R' is a member selected from the group consisting of hydrogen, a tissue targeting moiety (TM) or a cell membrane fusion moiety (FM) as described herein with the proviso that a mixture of hydrogen, targeting moieties and cell fusion moieties may be found on the same polymer backbone and/or within the polymer composition;

(3) X is a linker having the formula:

-Q-Z-Q'-

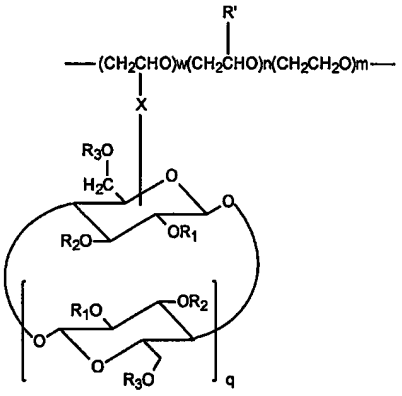
where Q is covalently bonded to the hydrophilic polymer chain either directly or by means of a pendant alkyl or other functional group and Q' is covalently bonded to the cyclodextrin. Q and Q' are independently members selected from the group consisting of NR_4, S, O, CO, CONH, and COO. In other words Q and Q' can comprise amine, alkylamine, acylamine, thio, ether, carbonyl, amide or ester moieties. Z comprises a member selected from the group consisting of an alkylene disulfide, $[-(CH_2)_aS-S(CH_2)_a-]$, alkylene $[-(CH_2)_a-]$, alkylene oxide $(-[(CH_2)_aO]_b(CH_2)_a-)$, or a short chained peptide where a is an integer of 1 to 10 and b is an integer of 1 to 20. Preferably Q is an amide and Q' is an amine, alkyl amine or acyl amine and the linker has the formula: -CONH-Z-NR_4-. Most preferably Q will be attached to a derivatized polymer chain through an alkylene $(-CH_2-)_a$ group. When Z is an alkylene disulfide, alkylene oxide or peptide, the linker is biodegradable. When Z is alkylene, the linker is non-biodegradable;

(4) R_1, R_2, R_3 and R_4 are independently members selected from the group consisting of H, alkyl ($C_{n'}H_{2n'+1}$), alkenyl ($C_{n'+1}H_{2(n'+1)-1}$) or acyl ($C_{n'}H_{2n'+1}CO$) where n' is an integer of 1 to 16, preferably 1 to 8, most preferably 1 to 4. When R_1, R_2, R_3 and R_4 are H, the cyclodextrin is more hydrophilic in nature. When one or more of R_1, R_2, R_3 and R_4 are alkyl, alkenyl or acyl groups, the derivatized cyclodextrin becomes more hydrophobic in nature. Therefore, when each of R_1, R_2, R_3 and R_4 is alkyl, alkenyl or acyl, the cyclodextrin is most hydrophobic. The acyl derivatized cyclodextrins are more biodegradable than the alkyl or alkenyl derivatized cyclodextrins;

(5) q is an integer of 5, 6 or 7, which makes the pendant cyclodextrin moiety to be α-, β-, or γ- cyclodextrin derivative, respectively. Preferably q is 6 or 7, and most preferably q is 6. In other words, the preferred cyclodextrin is a β-cyclodextrin;

(6) w is an integer such that each polymer backbone contains between 1.5 and 30 and preferably between 2 and 15 cyclodextrin moieties per 20 KD of polymer backbone. The integer "w" represents an average of cyclodextrin moieties in a polymeric composition since a polymeric composition is a mixture of polymer chains where each polymer in the chain may be variable in length, molecular weight and number of cyclodextrin moieties. Hence, each polymer has a weight average molecular weight and an average number of cyclodextrin moieties per 20 KD of polymer backbone within such polymeric composition.

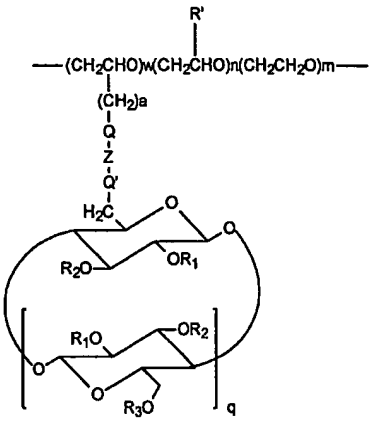
One embodiment of the present invention is a new class of CD-grafted-biocompatible polyethylene glycol (PEG) polymer which can be represented by Formula 2 as follows:



where q, w, X, R, R_1, R_2, R_3 and R_4 are as described in Formula 1, m and n are integers sufficient that when combined with w they represent a polyethylene oxide polymeric chain having the molecular weight as described for the hydrophilic polymer in Formula 1. In other words, as noted in Formula 1, the molecular weight of the biocompatible polyethylene oxide hydrophilic polymer backbone is preferably within the range of 5,000 to 1,000,000, more preferably within the range of 5,000 to 70,000 and most preferably within a range of 20,000 to 40,000. As noted in reference to Formula 1, the CDs can be grafted to the polymer by a single arm linker X via the 2, 3 or 6 positions of the CD molecule and, preferably, are grafted via 6 position of the CD molecule. While w has the same numerical value as in Formula 1 it is to be

noted that w is used to denote the number of cyclodextrin units per 20K of polymer backbone and does not refer to a polymeric unit containing "w" consecutively joined polyethylene glycol (CH_2CHXO) monomers. In other words, the polymer backbone contains "w" monomer units containing a pendent cyclodextrin which are spaced along the polymer backbone. The spacing may be random or uniform depending upon the synthesis.

Most preferably, the cyclodextrin containing polymers, are polyethylene glycol polymer backbones containing pendant CDs having following Formula 3 as follows:



where Q, Q', Z, R, R_1, R_2, R_3, R_4, a and q are as described in Formula 1, w is an integer such as to provide between 1.5 and 30 cyclodextrin units, and preferably between 2 and 15 cyclodextrin units per 20 KD polymer chain, as an average, m and n is integers sufficient that when combined with w they represent a polyethylene oxide polymeric chain having the molecular weight as described for the hydrophilic polymer in Formula 1. As explained for Formula 2, the monomeric polyethylene glycol units containing the pendent cyclodextrin are not consecutively joined and may be randomly or uniformly spaced along the polymer backbone.

Specific β-cyclodextrin co-polymers falling within the scope of Formula 3 are listed in Table 1.

Table 1

Comp No.	CD Polymer ID	w	Q	Z	Q'	R_1	R_2	R_3	R_4
6	PEG-SS-CD	5	C(O)NH	SS	NR_4	H	H	H	H
13	PEG-C3-CD	4.5	C(O)NH	C3	NR_4	H	H	H	H
18 (a) 18 (b)	PEG-L8-CD	5.5 8.5	C(O)NH	L8	NR_4	H	H	H	H
7	PEG-SS-DECD	1.5	C(O)NH	SS	NR_4	C_2H_5	H	C_2H_5	C_2H_5
11	PEG-GFLG-DECD	4.5	C(O)NH	GFLG	NR_4	C_2H_5	H	C_2H_5	C_2H_5
14	PEG-C3-DECD	2.6	C(O)NH	C3	NR_4	C_2H_5	H	C_2H_5	C_2H_5
20	PEG-L8-DECD	3.9	C(O)NH	L8	NR_4	C_2H_5	H	C_2H_5	C_2H_5
3	PEG-SS-AcCD	5	C(O)NH	SS	NR_4	CH_3CO	CH_3CO	CH_3CO	CH_3CO
15	PEG-C3-AcCD	4.5	C(O)NH	C3	NR_4	CH_3CO	CH_3CO	CH_3CO	CH_3CO
19 (a) 19 (b)	PEG-L8-AcCD	5.5 8.5	C(O)NH	L8	NR_4	CH_3CO	CH_3CO	CH_3CO	CH_3CO
16	PEG-C3-BnCD	4.5	C(O)NH	C3	NR_4	C_3H_7CO	C_3H_7CO	C_3H_7CO	C_3H_7CO

In Table 1 SS is $-CH_2CH_2SSCH_2CH_2-$, C3 is $-CH_2CH_2CH_2-$, L8 is $-CH_2CH_2OCH_2CH_2OCH_2CH_2-$ and GFLG is the tetrapeptide Gly-Phe-Glu-Gly.

These novel CD-grafted polymers of the present invention have the following advantages over their monomer precursors as drug carriers.

First, they have increased water solubility and reduced toxicity. Polyethylene glycol (PEG) is a linear polyether diol with many useful properties, such as good solubility, biocompatibility due to minimal toxicity, immunogenicity, and

antigenicity, and good excretion kinetics. These features have made PEG the most extensively studied drug carrier in pharmaceutical research which eventually lead to its FDA approval for internal administration. Therefore PEG can change the physical-chemical properties and toxicities of conjugated cyclodextrins to make them more biocompatible.

In addition, these CD-grafted polymers also provide enhanced guest molecule binding stability. Hydrophobic modification of CDs provides for a more hydrophobic interior and exterior of the cyclodextrin cavity and so increases the stability of inclusion complexes. Moreover multiple CDs in one polymer backbone will increase local CD concentration and produce cooperation in drug binding. Therefore, an amphiphilic co-polymer may form a polymeric micelle after binding to appropriate guest drugs through extra hydrophobic interactions or ionic interactions. Furthermore, these drugs containing CD-grafted polymers can be absorbed by cells through pinocytosis rather than by passive diffusion.

Moreover, the CD-grafted polymer can be used for the controlled-release and targeted-delivery of a bioactive agent. The polymer is likely to form a special type of polymeric micelles with appropriate drugs. Passive drug targeting can increase drug efficiency by targeting specific cells or organs, therefore reducing accumulation of the drug in healthy tissues and minimizing its toxicity thereby allowing higher doses to be administered, if needed. Following intravenous administration, polymeric micelles have been found to have a prolonged systemic circulation time due to their small size and hydrophilic shell, which minimizes uptake by the mononuclear phagocyte system (MPS), and to their high molecular weight which prevents renal excretion. Polymeric micelle-incorporated drugs may accumulate in tumors to a greater extent than the free drug and show reduced distribution into non-targeted areas such as the heart [Kwon et al, *J Control Rel*, 29, 17-23 (1994)]. Accumulation of polymeric micelles in malignant or inflamed tissues may be due to increased vascular permeability and impaired lymphatic drainage (enhanced permeability and retention (EPR) effect. The EPR effect is considered as a passive targeting method, but drug targeting could be further increased by binding to targeting moieties such as antibodies or sugars or by introducing a polymer sensitive to variation in temperature or pH. Targeting micelles

or pH sensitive micelles can serve for the delivery of drug to tumors, inflamed tissues or endosomal compartments, since they all are associated with a lower pH than normal tissue [Litzinger et al, *Biochim Biophys Acta*, 1113 (2), 201-27 (1992); Tannock et al, *Cancer Research*, 49 (16), 4373-84 (1989); Helmlinger et al, *Nat Med* 3 (2), 177-82 (1997)].

PEG is commercially available in a variety of molecular masses at low dispersity (Mw/Mn< 1.1). Based on their molecular size, they are arbitrarily classified into low molecular weight PEG (Mw<20,000) and high molecular weight PEG (Mw>20,000). Most recent applications of PEG are focused on the attachment of cytotoxic anticancer drugs to the PEG or the grafting of PEG to proteins, micelles or liposomes which leads to a reduction in systemic toxicity, longer retention time within the body, alteration in biological distribution, and improvements in therapeutic efficacy [Takakura et al, Crit Rev Oncol, Hematol 18(3), 207-31 (1995); Duncan et al, *Anticancer Drugs*, 3 (3), 175-210 (1992)]. A recent study found that the renal clearance of PEG decreased with an increase in molecular weight, with the most dramatic change occurring at a MW of 30,000 after i.v. administration. The half-time (t1/2) of PEG circulating in blood also showed a concomitant and dramatic increase. For instance, the t1/2 for PEG went from approximately 18 min to 16.5 hour as the molecular weight increased from 6,000 to 50,000. Consequently, conjugation of anticancer drugs with PEG of a molecular weight of 20,000 or greater can prevent rapid elimination of the PEG-conjugated species and allow for passive tumor accumulation [Greenwald et al, *Crit Rev Ther Drug Carrier Syst* 17 (2), 101-61 (2000)].

In one embodiment of the present invention, a carboxyl group grafted PEG (20,000 Daltons or 25,000 Daltons containing 8 to 10 carboxyl groups per PEG molecule) is used as the starting material to conjugate with the cyclodextrins. In order to keep the steric hindrance effect to a minimum, CD moieties were conjugated at the small open end (6-position) of their cavity to the PEG backbone through one of the 7 primary hydroxyl groups. In addition, a flexible linear linker was used to keep the CD moiety away from the polymer backbone and allow it to move freely. Due to the biocompatibility of the materials and pliability of the polymers of the present

invention, they will cause minimal toxicity and minimal mechanical irritation to the surrounding tissue.

A dosage form comprised of a solution of the grafted polymer that contains either dissolved drug or drug as a suspension or emulsion is administered to the body. The only limitation as to how much drug can be loaded into the formulation is one of functionality, namely, the drug load may be increased until the desired properties of the polymer are adversely affected to an unacceptable degree, or until the properties of the formulation are adversely affected to such a degree as to make administration of the formulation unacceptably difficult. Generally speaking, it is anticipated that in most instances the drug will make up between about 0.01% to 50% by weight of the formulation with ranges of between about 0.1% to 25% being most common. These ranges of drug loading are not limiting to the invention. Provided functionality is maintained, drug loadings outside of these ranges falls within the scope of the invention.

A distinct advantage to the compositions of the subject of this invention lies in the ability of the grafted polymer to increase the solubility and stability of many drug substances. The combination of hydrophobic CDs and hydrophilic polymers renders the polymer amphiphilic in nature. In that regard it functions much as a combination of cyclodextrin inclusion and polymeric micelle system. This is particularly advantageous in the solubilization of hydrophobic or poorly water soluble drugs such as cyclosporin A, tacrolimus, saquinavir and paclitaxel.

Another advantage to the composition of the invention lies in the ability of the polymer to increase the chemical stability of many drug substances. Various mechanisms for the degradation of drugs have been observed to be inhibited when the drug is in the presence of the polymer. For example, paclitaxel and cyclosporin A are substantially stabilized in the aqueous polymer composition of the present invention relative to certain aqueous solutions of these same drugs in the presence of organic co-solvents. This stabilization effect on paclitaxel and cyclosporin A is but illustrative of the effect that can be achieved with many other drug substances.

The drug loaded CD-grafted polymers of the present invention may be administered via various routes including parenteral, topical, transdermal,

transmucosal, inhaled or inserted into a body cavity such as by ocular, vaginal, buccal, transurethral, rectal, nasal, oral, pulmonary and aural administration.

This invention is applicable to bioactive agents and drugs of all types including nucleic acids, hormones, anticancer-agents, and it offers an unusually effective way to deliver polypeptides and proteins. The only limitation to the polypeptide or protein drug which may be utilized is one of functionality. In some instances, the functionality or physical stability of polypeptides and proteins can also be increased by addition of various additives to aqueous solutions or suspensions of the polypeptide or protein drug. Additives, such as polyols (including sugars), amino acids, surfactants, polymers, other proteins and certain salts may be used. Developments in protein engineering may provide the possibility of increasing the inherent stability of peptides or proteins. While such resultant engineered or modified proteins may be regarded as new entities in regards to regulatory implications, that does not alter their suitability for use in the present invention.

In addition to peptide or protein based drugs, other drugs from all therapeutic and medically useful categories may be utilized. These drugs are described in such well-known literature references as the Merck Index, the Physicians Desk Reference, and The Pharmacological Basis of Therapeutics.

Paclitaxel is a diterpeniod natural product showing encouraging activity against ovarian, breast, head, and non-small-cell lung cancers. Recently it has been approved in the form of paclitaxel for treatment of breast and refractory human cancers. One of the major problems with paclitaxel is its extremely low aqueous solubility. The present formulation of this drug contains 30 mg of paclitaxel in 5 ml of a 50/50 mixture of Cremophore EL (polyethoxylated casteror oil, a solubilizing surfactant) and ethanol. When diluted in saline, as recommended for administration, the concentration of paclitaxel is 0.6-1.2mg/ml (0.7-1.4 ml). The diluted solution is expected to contain mixed "micelle" particles of Paclitaxel/Cremophor and has been reported to be physically unstable over time, because dilution to some concentrations apparently yields supersaturated solutions. In addition, Cremophor, a non-charged surfactant, has been reported to cause histamine release and to be associated with adverse effects such as severe allergic reactions [Sharma et al, *Int J Cancer*, 71 (1),

103-7 (1997)]. Cyclodextrin derivatives have been examined to see if they can solubilize paclitaxel. It was found that methylated cyclodextrins worked much better than other hydrophilic cyclodextrin derivatives in improving the water solubility of paclitaxel (at 50% CD concentrations, HPCD and DMCD could dissolve about 0.7 and 33 mg/ml paclitaxel respectively) [Sharma et al. *J Pharm Sci*, 84 (10), 1223-30 (1995)]. However, the toxicity of DMCD and the high concentration needed to complex therapeutic levels of paclitaxel limit its clinical application. The CD-grafted amphiphilic polymers of the present invention provide significant advantages over prior art formulations facilitated by ease of preparation and administration, lowered toxicity, rapid and controlled release of active agents and targetable delivery.

Antisense oligonucleotides and their analogs , such as peptide DNA (PNA), morpholino-DNA, P-ethoxy DNA, methylphosphonate-DNA, etc., have been shown to have great applications in biomedical research, but their pharmaceutical applications have been largely limited by their stability and/or solubility, and cell uptake behavior. Currently there is no effective means to safely deliver intact antisense oligonucleotides to their target sites *in vivo*. And this is particularly true for their neutral analogs, such as PNA, morpholino DNA, P-ethoxy DNA and methylphosphonate-DNA, because they cannot efficiently bind to any of the current antisense oligonucleotide carriers which are mostly poly- cationic polymers. However, the CD-grafted amphiphilic polymers of the present invention can be effective carriers of neutral anologs because every nucleoside unit has an aromatic base residue which is a potential target to be included by the cyclodextrin, thus the CD-grafted polymers can bind oligonucleotides and their analogs through enhanced CD inclusion mechanisms. This binding can be very strong due to cooperation between the multiple CD moieties on the polymer and the multiple aromatic base rings on antisense oligonucelotides. In addition, extra ionic interactions (for charged oligonucleotide) or hydrophobic interactions (for non-charged oligonucleotide analogs) can also strengthen the binding between antisense oligonucelotides and CD-polymer carriers. Eventually the final binding complex may form a loose or tight polymeric micelle depending on their content, and therefore can safely deliver antisense oligonucleotides and their neutral analogs to cells.

In summary, the CD-grafted polymers of the present invention improve the drug/binding complex stability via multiple CD moiety co-operations and external hydrophobic or ionic interactions. It is likely that inclusion is an essential mechanism for the drug binding capability of the polymers of the present invention. However, ionic interactions and external hydrophobic interactions (outside the CD cavity) may also make significant contributions depending on the molecular structures of the specific co-polymers and guests. Furthermore, appropriately constructed PEG-CD co-polymers of the present invention are excellent paclitaxel solubilizers and carriers for safe therapeutic application. They can also be used as solubilizers and carriers for other hydrophobic drugs. The CD-grafted amphiphilic polymers of the present invention are water soluble and biocompatible, and have very slow release kinetics, especially when they contain high weight ratios of hydrophobic moieties. In addition, the strong binding constant of the drug/polymer complexes makes for slow release of the bound drug upon dilution, and it sometimes even needs replacement by other molecules. Therefore they may be used as ingredients in oral formulations for delivery of certain water soluble drugs.

Furthermore, properly constructed CD-grafted polymers of the present invention can be used to deliver antisense oligonucleotides and their non-charged analogs, as well as hydrophobic peptides and proteins, since external hydrophobic interactions may produce enough stability for hydrophobic antisense oligonucleotides or hydrophobic peptides. The negatively charged oligonucleotides are also expected to be good guest molecules for some specially constructed polymers, because a basic nitrogen in the linker of the polymer could neutralize negative charge under appropriate conditions.

The following Examples are presented to illustrate the process of preparing the composition and method of using the composition of the present invention.

Example 1

Materials and methods: PEG with pendant propionic acid groups (PEG-10PA and PEG-8PA, Mw = ~20 KD, SunBio, Inc., Anyang City, South Korea) was dried overnight in vacuo at room temperature. β-Cyclodextrin (TCI America, Portland, OR)

was dried in vacuo at 130 °C overnight before use. Other chemicals were from Aldrich Chemical Company, Inc. of Milwaukee, WI) and used as received without further purification. HPLC analysis was performed on a Waters system equipped with RI detector and Ultrahydrogel 120 and Ultrahydrogel 500 SEC columns. ^{1}H-NMR was recorded on a Varian 400 MHz machine.

Synthesis of PEG-SS-CD (Compound 2)

Mono-6-(6-amino-3,4-dithio-hexylamino)-6-deoxy--β-cyclodextrin (Compound 1):

Cystamine dihydrochloride (1.0 g, 4.44 moles, Fw = 225.2) was dissolved in 30 ml distilled water, followed by addition of 1.0 M KOH (8.88 moles) and mono-6-tosyl-β-cyclodextrin (0.5g, Fw = 1289) powder. The resulting suspension was stirred in a 70 °C oil bath overnight, then concentrated to about 4 ml. The mixture was applied on a Sephadex G-25 column (2.5 X 80 cm), eluted with 0.1 M TEA. About 0.38g compound 1 was obtained.

PEG-SS-CD (compound 2):

Carboxyl group grafted PEG (2.24 g, PEG-8PA, 20kDa, polyethylene glycol containing ~8 pendant propionic acid groups with average molecular weight of ~20,000) was dissolved in 25 ml anhydrous DMF, the mixture was cooled to 0 °C on ice under protection of argon. To this was added 280 ul of tributylamine (1.18 mmoles, Fw = 185.36, d = 0.778), followed by 175 ul of isobutylchloroformate (IBCF, Fw = 136.6, d = 1.053) in 1 ml DMF. The mixture was stirred at 0 °C for 1 hour. The reaction mixture was then slowly added to a solution of 1.75 g compound 2 in 100 ml DMF at room temperature. After being stirred at room temperature overnight, the reaction was stopped by addition of 1 ml water. The mixture was concentrated and then diluted with 60 ml water. The product solution was purified on a Sephadex G-50 column, eluted with 0.1 M TEA followed by ether precipitation. ^{1}H-NMR analysis indicated that about 5 CD moieties were conjugated to a PEG backbone having a molecular weight of about 20,000 Daltons. The retention time of the product is about 0.45 minute later than that of the starting PEG as determined by

HPLC chromatography [GPC column, Rt (product) = 17.33', vs. Rt (PEG-8A) = 16.87']. ^{1}H-NMR (400 MHz, D2O): δ, 5.0 (s, 7H, H1'), 3.3-3.9 (m, 370H, 41H-CD, 329H-PEG).

Example 2

Synthesis of PEG-SS-AcCD (compound 3)

PEG-SS-CD (compound 2, 1.0g, ~5 CDs / 20 kD-PEG) was dried in a P_2O_5 desiccator, followed by co-evaporation with 50 ml anhydrous pyridine. The residue was dissolved in 30 ml pyridine under protection of argon, followed by addition of 2.0 ml acetic anhydride (Fw = 102.1, d = 1.08). The mixture was dried on a rotary-evaporator after being stirred at room temperature for 2 days. The crude product was purified by repeated ether precipitation from methanol. HPLC (GPC) analysis showed a 0.46 minute time delay of the product compared to the starting polymer (Rt = 19.70' of the product vs. Rt = 19.24' of the reactant polymer). ^{1}H-NMR analysis indicates that each 20 kD PEG contains about 5 CD moieties and all hydroxyl groups are acetylated. ^{1}H-NMR (400 MHz, D_2O): δ, 4.7-5.5(s, 14H, H1', H3'), 3.4-5.5 (m, 382H, 35H-CD, 347H-PEG), 2.05 (m, 20H, H-Ac).

Example 3

Synthesis of PEG-SS-DECD (compound 7)

PEG-SS-NH2 (compound 4):

Carboxyl group grafted PEG (PEG-8PA, 2.6 g, ~2.0 mmole COOH group) was dissolved in 30 ml anhydrous DMF and cooled to 0 °C on ice. To this was added tributylamine (0.35 ml,1.5 mmoles, Fw = 185.36, d = 0.778), followed by the addition of isobutyl chloroformate (0.20 ml, 1.5 mmoles, Fw = 136.6, d = 1.053). The mixture was stirred at 0 °C for 80 minutes and was carefully added to a solution cystamine (3.5 g, Fw = 152.2, 23 mmoles) in 50 ml anhydrous DMF. The mixture was stirred at room temperature for 20 hours, concentrated to about 20 ml on rotary evaporator at 40 °C, then dialysed against distilled water (4 X 5 L over 26 hours, Sigma D-0655, MWCO = 12,000) after being diluted with 50 ml water. The dialysis solution was concentrated by rotary evaporation at 40 °C, obtaining 4.1 g of syrup. The syrup was

dissolved in 10 ml methanol, then precipitated by addition of 80 ml ethyl ether. The precipitate was collected by centrifugation and this precipitation process was repeated twice. The final product was a white powder, weighing about 2.2 g. The product showed only one nice peak in its HPLC (GPC) chromatogram, and the retention time (18.66') was about 1.5 minutes longer than that of the starting PEG-8PA (17.11').

N-(β-Cyclodextrin-6-yl) glycine methyl ester (compound 5):

Glycine methyl ester hydrochloride (1.5 g, Fw = 125.56, 12 mmoles, from Aldrich) was dissolved in 100 ml anhydrous DMF with protection of argon. To this was added DIPEA (2.1 ml, 12 mmoles, Fw = 129.25, d = 0.724), followed by the addition 6-mono-tosyl cyclodextrin powder (3.0 g, Fw = 1289, ~80% pure, ~1.8 mmoles). The mixture was stirred at room temperature to a clear solution. The temperature was slowly raised to about 70 °C followed by another 4 hour stirring. The mixture was then concentrated to a syrup on a rotary evaporator at 55 °C. The crude product was dissolved in 40 ml hot water, precipitated by adding ~80 ml acetone after cooled to room temperature. The white precipitate was collected by filtration and dried in a vacuum overnight. About 2.3 g of the desired compound 5 was obtained. This product was used in next step without further purification.

N-(Heptakis-2-O-ethyl-6^B,6^C,6^D,6^E,6^F,6^G-hexa-O-ethyl-β- Cyclodextrin-6^A-yl)-glycine (compound 6):

N-(β-Cyclodextrin-6-yl) glycine methyl ester (compound 5, about 2.0 g, Fw = 1206, ~1.6 mmoles) was dissolved in 15 ml DMSO and 15 ml DMF. The solution was cooled to 0 °C in an ice bath, followed by addition of 10 g BaO and 10 g $Ba(OH)_2.H_2O$ with the protection of argon. To this white suspension was slowly added 20 ml diethyl sulfate. The mixture was stirred at 0 °C for 1 hour, followed by another 24 hour stirring at room temperature. Another 20 ml of diethyl sulfate was slowly added within an hour, followed by another 24 hour stirring at room temperature. To the viscous reaction mixture was slowly added 60 ml 5 N NaOH at 0 °C, then the mixture was stirred at room temperature for one hour. It was extracted

with 2 X 200 ml of chloroform. The combined organic phase was concentrated to a wax product after drying with Na_2SO_4. The crude product was dissolved in 20 ml methanol, followed by addition of 20 ml of distilled water. The mixture was filtered in vacuum to remove the trace amount of precipitate. The clear filtrate was concentrated to get an orange foam solid (about 1.8 g), which contained about 50% of the desired compound 6. This crude product was directly used in the next reaction after being dried overnight in vacuum P_2O_5 desiccator.

PEG-SS-DECD (compound 7):

The crude compound 6 (1.4 g, ~0.46 mmole) was dried by co-evaporation with 2 X 20 ml anhydrous DMF, then re-dissolved in 20 ml DMF followed by addition of 0.19 ml tributylamine (0.8 mmole, Fw = 185.36, d = 0.778). The mixture was cooled to 0 °C on ice. To this cold solution was slowly added isobutyl chloroformate (60 ul, 0.46 mmole, Fw = 136.6, d = 1.053) in 2 ml DMF. The mixture was stirred at 0 °C for 1.5 hours, then transferred to a solution of PEG-SS-NH2 (compound 4, 0.4g) in 10 ml anhydrous DMF at room temperature, followed by addition of DIPEA (28 ul, 0.16 mmole, Fw = 129, d = 0.724). The mixture was concentrated to a syrup after overnight stirring at room temperature. The syrup was triturated with 30 ml ethyl ether to produce an orange precipitate. The precipitate was collected by filtration and washed with ethyl ether. The solid was further purified twice by ether precipitation from methanol. About 0.55 g light orange solid was obtained. ^{1}H-NMR indicated the product was the desired PEG-SS-DECD product, but only about 1.5 CD moieties were conjugated onto a 20-KD PEG molecule and about 13 ethyl groups per cyclodextrin. ^{1}H-NMR (400 MHz, D2O): δ, 5.1 (7H, m, H1' and H3'), 3.2-3.9 (m, 1041H, 41H-CD, 1000 H-PEG), 2.78 (m, 30H, CH2-Et), 1.15 (b, 45, CH3-Et).

Example 4

Synthesis of PEG-GFLG-DECD (compound 11)

Mono-6-(N^3-Boc-3-amino-propylamino)-6-deoxy-β-Cyclodextrin (compound 8):

Mono-Boc-1,3-diamino-propane (3.5g, ~3.0 moles, Prepared according to the methods described by Jean Francois Pons et. al., Eur. J. Org Chem, 1998, 853-859)

was dried by co-evaporation with 2.8 ml (12 mmoles, Fw = 185.36, d = 0.778) tributylamine and 30 ml anhydrous DMF twice. The final dried oil was mixed with 100 ml anhydrous DMF, followed by addition of DIPEA (2.1 ml, 12 mmole, Fw = 129, d = 0.742). To this solution was added 3.5 g of 6-mono-tosyl-6-O-β-cyclodextrin. The mixture was stirred at room temperature to the complete dissolution of the solid. Then the mixture was stirred overnight at 70 °C in an oil bath. The mixture was concentrated to about 10 ml on a rotary evaporator at 45 °C, then precipitated with 100 ml of acetone. The white precipitate was collected by filtration, washed with acetone. About 3.2 g of product was obtained. It contained about 60% of the desired compound 8 as estimated on a TLC (Rf = 0.12, Silica gel, developed in 80 : 10 : 10 / AcOH : $CHCl_3$: H_2O, stained with 5% phosphomolybdic acid in 95% ethanol). This product was directly ethylated in the next step.

Mono-(Heptakis-2-O-ethyl-6^B,6^C,6^D,6^E,6^F,6^G-hexa-O-ethyl-β- Cyclodextrin-6^A-yl)-1,3-diamino-propane (compound 9):

Mono-6-(N^3-Boc-3-amino-propylamino)-6-deoxy-β-Cyclodextrin (Compound 8, 3.0g) was dissolved in 40 ml anhydrous DMF and 40 ml DMSO at 0 °C, then mixed with 10 g of BaO and 10 ml of $Ba(OH)_2.H_2O$ under protection of argon. The mixture was cooled to 0 °C, then 20 ml of diethyl sulfate was slowly added. The mixture was stirred at 0 °C for 6 hours followed by another 2 days at room temperature. To the reaction mixture was added 25 ml of cold ammonia followed by another 3 hour stirring at room temperature. The final reaction mixture was diluted with 50 ml H_2O, extracted with 3 X 100 ml ethyl acetate. The organic phase was thoroughly washed with 2 X 200 ml saturated $NaHCO_3$ and 3 X 200 water, then concentrated after drying with sodium sulfate. About 2.8 g of orange solid was obtained after being dried in vacuum overnight. The product was dissolved in 10 ml of trifluoroacetic acid. The clear solution was stirred at room temperature for 3 hours, then 15 ml of water was added. The mixture was stirred at room temperature for another 20 minutes, then dried on a rotary evaporator at 45 °C. The residue was dissolved in 150 ml ethyl acetate, washed with 3 X 100 ml saturated $NaHCO_3$ and 100 ml of saline. The organic phase was concentrated after being dried with Na_2SO_4.

About 2.0 grams of crude compound 9 was obtained. This product was directly used in the next conjugation reaction.

PEG-GFLF-DECD (Compound 11):

PEG-GFLG (tetrapeptide Gly-Phe-Leu-Gly grafted PEG polymer, compound 10, ~4.5 GFLG peptide in a PEG of 20,000 prepared from PEG-8PA and GFLG peptide) (2.0 g, ~0.4 mmole –COOH, dried by co-evaporation with 30 ml DMF) was dissolved in 30 ml anhydrous DMF and 0.17 ml of tributylamine (0.7 mmole, Fw = 185.36, d = 1.053) with protection of argon. To this was added 0.078 ml (0.6 mmole) isobutylchloroformate in 2 ml DMF after cooling to 0 °C. The mixture was stirred at 0 °C for 1.5 hours, then slowly added to the solution of 2.0 g compound 9 in 20 ml DMF at room temperature, followed by addition of 0.087 ml of DIPEA (0.5 mmole). The mixture was stirred at room temperature overnight, concentrated to about 10 ml, precipitated with 90 ml of cold ethyl ether. The orange precipitate was collected by filtration and was further precipitated 3 times using ether from methanol. The final product was about 2.2 grams. The retention time of the product (Rt = 18.42') was 0.67 minutes longer than that of the starting PEG-GFLG polymer (Rt =17.76') on HPLC (GPC) chromatography. ^{1}H-NMR indicates the product is the desired compound 11: every 20kD polymer contains about 4.5 tetrapeptide GFLG and 1.8 CD moieties and every CD moiety has about 13 ethyl groups. ^{1}H-NMR (400 HMz, D_2O): δ, 7.20 (5H, m, ArH-Phe), 5.1 (2.8H, m, H1'-CD), 3.0-4.0 (645H, m, 41H-CD, 574H-PEG, 30H-Et), 1.1 (15.6H,m, 30H, CH3-Et), 0.9 (6H, d, CH3-Leu).

Example 5

Synthesis of PEG-C3-AcCD, PEG-C3-DECD and PEG-C3-BnCD

Mono-6-(γ-amino-propanyl-amino)-6-deoxy-β-cyclodextrin (compound 12):

Mono-6-tosyl-6-deoxy-cyclodextrin (6.5 g, Fw = 1269) was dissolved in 200 ml of anhydrous DMF and 60 ml of diaminopropane under vigorous stirring at room temperature. The clear mixture was stirred at room temperature for 2 hours followed by another 20 hours at 65 °C C. The mixture was concentrated to about 20 ml at 45 °C C. To this was added 200 ml of cold isopropanol at room temperature. The white

precipitate was collected by filtration. The solid was re-dissolved in 25 ml water and 25 ml TEA. To this was slowly added 300 ml of acetone at 0 °C C. The precipitate was collected by filtration, and re-precipitation was repeated two-more times. The final product was about 5.5 grams. It contains about 80% of the desired compound 12 and about 20% free cyclodextrin. The product was used for the next reaction without further purification.

PEG-C3-CD (compound 13): Mono-6-(γ-amino-propanyl-amino)-6-deoxy-β-cyclodextrin (compound 12, 6.2 g) was conjugated to PEG-8PA (4.1 g) using the same method as described in the synthesis of PEG-SS-CD. About 4.3 g of pure product was obtained after GPC purification. The retention time of the product (17.87') is 0.76 minute longer than that of starting PEG-8PA (17.11'). ^{1}H-NMR indicates the product is the desired compound 13, which contains about 4.5 CD moieties in every 20 KD PEG molecule. ^{1}H-NMR (400 HMz, D2O): δ, 5.0 (7H, s, H1'-CD), 3.4-3.9 (412H, m, 41H-CD, 371H-PEG).

PEG-C3-AcCD (compound 15): PEG-C3-CD (1.0 gram, ~4.5 CDs / 20 KD PEG) was acetylated using the same method as described in the preparation of PEG-SS-AcCD. About 1.0 gram of product was obtained and its retention time (17.99') was only about 7.2 seconds longer than that of the starting polymer (PEG-C3-CD, 17.87'). However ^{1}H-NMR indicated that the product is the desired compound 15: the polymer contains 4.5 of CD moieties in every 20 kD PEG and bout 90% of the hydroxyl groups on the pendent CDs were acetylated. ^{1}H-NMR (D2O): δ, 4.9-5.4 (14H, m, H1'-CD and H3'-CD), 3.2-4.5 (m, 490H, 35H-CD, 455 H-PEG), 2.03 (d, 64H, CH_3CO-).

PEG-C3-BnCD (compound 16): PEG-C3-CD (Compound 13, 0.9g, ~4.5 CDs / 20 KD PEG) was dried by co-evaporation with 20 ml anhydrous pyridine and then re-dissolved in 30 ml pyridine with protection of argon. To this was slowly added 3 ml of butyryl chloride (Fw = 106.55, d = 1.026) at room temperature (cooled with ice as the reaction temperature went up). Methanol (5.0 ml) was added after the mixture was

stirred at room temperature for 4 hours, followed by another 30 minutes of stirring at room temperature. The mixture was concentrated on a rotary evaporator to a wax solid. The solid was dissolved in 20 ml of methanol, and diluted with 20 ml water. The clear solution was dialyzed against 2 X 5 L 20% isopropanol/water. The opaque dialysis solution was concentrated in a Speed-Vac at room temperature. The pellet was further precipitated three times from methanol using ether. The product is practically insoluble in water, but very soluble in methanol or chloroform. Yield = 90%. ^{1}H-NMR indicates that the product is the desired compound 16: about 80 % of the hydroxyl groups on the pendant cyclodextrins were butyrylated. ^{1}H-NMR ($CDCl_3$): δ, 4.6-5.3 (14H, m, H1' and H3'), 3.2-4.5 (m, 541, 35H-CD, 486H-PEG), 2.30 (m, 36H, $CH_3CH_2CH_2CO$-, 1.65 (m, 36H, $CH_3CH_2CH_2CO$-), 0.95 (m, 54H, $CH_3CH_2CH_2CO$-).

Example 6

Synthesis of PEG-L8-AcCD and PEG-L8-DECD

Mono-6-(8-amino-3,6-dioxy-octylamino)-6-deoxy--β-cyclodextrin (Compound 17):

In a 500 ml round bottom flask was charged with 2,2'-(ethylenedioxy)bis(ethylamine) (300 ml, Fw = 148) and mono-6-tosyl-β-cyclodextrin (24.4 g, Fw = 1269, dried in a P_2O_5 desiccator overnight) under the protection of argon. The suspension was stirred at room temperature to the complete dissolution of all of the solid (~1.0 hour). The mixture was stirred for another 4 hours at 75 °C. The reaction mixture was slowly poured into 1.8 L of cold isopropanol. The precipitate was collected by filtration and washed with isopropanol. The precipitate was dissolved in 200 ml warm water (50 °C), then slowly poured into 1.8 L of ice cold isopropanol with stirring. The precipitate was collected by filtration after being cooled to –20 °C. This isopropanol precipitation process was repeated two more times. About 24 grams of white powder was obtained. HPLC analysis (GPC, eluted with 0.1 M $NaNO_3$) showed that the product contains about 85% desired compound (Rt = 39.21') and ~15% non-modified β-CD (Rt = 32.25'), no free diamine reactant was detected. So this product was directly used for next conjugation. ^{1}H-NMR (400 HMz,

D2O): δ, 4.97 (7H, m, H1'), 3.7-3.9 (26H, m, 7H3', 7H5', 6H6', 6H6"), 3.3-3.6 (24H, m, 7H2/, 7H4', 1H6', 1H6", 8H-linker), 2.71 (4H, m, CH_2N-linker).

PEG-L8-CD (Compound 18):

PEG-8PA (4.0g, ~8-COOH / PEG-20K, ~1.7 mmoles COOH, dried in a P_2O_5 desiccator overnight and co-evaporated with 50 ml anhydrous DMF) was dissolved in 50 ml anhydrous DMF and 0.54 ml tributyl amine (TBA, Fw = 185.36, d = 0.778, 2.27 mmoles). The clear mixture was cooled on ice, then 0.29 ml isobutyl chloroformate (IBCF, Fw = 136.6, d = 1.053, 2.2 mmoles) was added at 0 °C. The mixture was stirred at 0 °C for 1 hour, and was then slowly added to a solution of mono-6-(8-amino-3,6-dioxy-octylamino)-6-deoxy--β-cyclodextrin (compound 17, 5.0 g , Fw = 1336, ~ 80% pure, ~2.6 mmoles, dried in a P_2O_5 desiccator overnight) in 50 ml anhydrous DMF at room temperature. After overnight stirring, the mixture was concentrated to about 20 ml on a rota-vap at 50 °C. The mixture was diluted with 60 ml of water and purified on a Sephadex-G-50 column (2.5 X 80 cm, eluted with 0.1 M TEAA, pH = 10.0, collected 8 ml / ml). The fractions were analyzed by GPC-HPLC and the polymer fraction was pooled into two parts: Part A: fraction 9 through 30; Part B: fraction 31 through 35.

Both parts were concentrated to wax solids on rotary evaporator and then re-dissolved in 15 ml methanol. The products were precipitated by 5 ml TEA and 120 ml of ethyl ether. The white precipitates were collected by filtration. Part A and Part B weighed 4.7 gram and 0.55gram, respectively. ^{1}H-NMR analysis confirmed both parts were the desired PEG-L8-CD product, but with different cyclodextrin loading: on average a 20 KD-PEG polymer contains about 5.5 and 8.5 cyclodextrin moieties in part A and part B, respectively. ^{1}H-NMR (400 MHz, D2O): δ, Part A: 5.0 (s, 7H, H1'), 3.3-3.9 (382H, m, 41H-CD, 12H-linker, 329H-PEG; Part B: 5.0 (s, 7H, H1'), 3.3-3.9 (256H, m, 41H-CD, 12H-linker, 203H-PEG).

PEG-L8-AcCD (compound 19):

PEG-L8-CD (Compound 18, 1.0 g, ~ 5.5 CDs/20KD PEG, dried in P2O5 desiccator overnight) was dried by co-evaporation with 40 ml anhydrous pyridine,

then re-dissolved in 40 ml anhydrous pyridine under protection of argon. To this was added 3.0 ml acetic anhydride. The mixture was stirred at room temperature for 2 days, concentrated to about10 ml on a rotary evaporator at 45 °C. To this was slowly added 90 ml of ethyl ether. The precipitate was collected by filtration. The product was further purified by ether precipitation three more times from methanol. The final white powder was dried in a vacuum, and it weighed 1.07 g. ^{1}H-NMR confirmed the product is the desired product 19: Every 20 kD PEG contains about 5.5 CD moieties and about 90% of the hydroxyl groups on the pendent CD moieties of the polymer were acetylated. ^{1}H-NMR (D2O): δ, 4.9-5.4 (14H, m, H1'-CD and H3'-CD), 3.2-4.5 (m, 422H, 34H-CD, 12H-linker, 376H-PEG), 2.03 (d, 64H, CH3CO-).

PEG-L8-DECD (compound 20):

PEG-L8-CD (compound 18, 1.0 g, ~5.5 CDs / 20 KD PEG, dried in P_2O_5 desiccator overnight) was dissolved in 5 ml anhydrous DMSO and 5 ml anhydrous DMF, the solution was cooled to 0 °C on ice under protection of argon. To this was added 0.75g BaO and 0.75 g $Ba(OH)_2.H_2O$ powder, immediately followed by addition of 3 ml of diethyl sulfate in three portions over a one hour period. The suspension was stirred at 0 °C for 2 hours, followed by stirring for another 2 days at 4 °C. Then 80 ml of cold ethyl ether was added at 0 °C, followed by another 30 minutes of stirring at 0 °C. The orange precipitate was collected by filtration and dissolved in 50 ml 50% methanol/water. The mixture was dialyzed (MWCO = 12,000) against 5 L of 0.01 N HCl, then 2 X 5L water. The final dialysis solution was concentrated, obtaining about 1 g of wax product. It was further purified by ether precipitation from methanol twice. ^{1}HNMR analysis indicated that about 4 CDs are present in every 20 KD-PEG, and each CD moiety carries about 11 ethyl groups. This means about 30% of the CD moieties came off the PEG backbone during the alkylation process. ^{1}H-NMR (400 HMz, D2O): δ, 4.9-5.3 (7H, m, H1'-CD), 3.1-4.0 (540H, m, 41H-CD, 469H-PEG, 8H-linker, 22H-CH2-ehthyl), 1.2 (33H, m, CH3-ethyl)

Thirteen representative cyclodextrin-grafted- PEG polymers (Table 1) have been prepared according to Examples 1-6 and FIG. 4-8, wherein the linkers are either biodegradable (X = -SS- or –GFLG-) or non-biodegradable (-C3- or –L8-). The

pendent cyclodextrin moieties are either natural β-CD (PEG-X-CD) or modified with hydrophobic groups including ethyl (PEG-X-DECD), acetyl (PEG-X-AcCD) or butyryl (PEG-C3-BnCD). GPC-HPLC was used to monitor each step of the preparation process, and it was found that all final polymer products had longer retention times than the corresponding PEG precursors. The structure of all of the product polymers were confirmed by ^{1}H-NMR analysis, it was found that their CD contents varied from an average of 1.5 CDs to 8.5 CDs on every 20 KD PEG backbone (Table 2). They are all highly soluble in most organic solvents (chloroform, methanol, ethanol, etc.). They are also highly soluble in water, except PEG-C3-BnCD.

Table 2. Structure characteristics of some cyclodextrin grafted PEG co-polymers

Polymer name	t_R (min*)	Number of CDs/ 20 Kd Polymer**	CD modification**
PEG-ss-CD	19.34	3.9	None
PEG-ss-AcCD	19.24	3.9	~100% acetylation
PEG-C3-CD	18.07	4.8	None
PEG-C3-AcCD	17.86	4.8	~80% acetylation
PEG-L8-CD (A)	18.12	4.6	None
PEG-L8-AcCD (A)	17.98	4.6	~95% acetylation
PEG-L8-CD (B)	18.43	5.9	None
PEG-L8-AcCD (B)	18.08	5.9	~84% acetylation
PEG-L8-CD (C)	18.71	5.4	None
PEG-L8-AcCD (C)	18.53	5.4	~100% acetylation
PEG-GFLG-DECD		2.5	~67% ethylation
PEG-L8-DECD	18.0	3.9	~67% ethylation

PEG-C3-BnCD	4.5	~80% Butyrylation

* GPC columns of Waters Ultrahydrogel (120 & 500), eluted with 0.1 M NaNO3;

** Calculated according to ^{1}H-NMR spectrum recorded on a Varian 400 HMz;

Example 7

Preparation of Paclitaxel complexes with CD polymers or CD monomers

(A) Co-dissolving method: This method is suitable for all complexes with water soluble polymers

The aqueous solution of polymer (or monomer controls) (usually about 100 mg/ml) was mixed with equal volume (usually 40 to 2000 ul) of the paclitaxel solution ($C_{paclitaxel}$ = 0.1 to 8.0 mg/ml in methanol). The mixture was incubated at room temperature for about half an hour. Then the solvents were removed in a centrifuge concentrator at room temperature. The concentrated syrup or wax solid was reconstituted by adding water or PBS buffer to the original volume. The mixture was usually a clear or slightly cloudy solution after 30 minutes of reconstitution. The un-dissolved paclitaxel particles were removed either by ultra-filtration (0.2 um filter) or by centrifugation (20 minutes at 20,800g and room temperature). The paclitaxel concentration in the clear supernatant was quantified by UV absorbance at 290 nm by using the corresponding cyclodextrin polymer solution as the background calibration.

(B) Dialysis method: This method is suitable for the preparation of all paclitaxel/polymer complex solutions:

The methanol solution of the polymer (usually 100 mg/ml) was mixed with equal volume (100 ul) of paclitaxel solution (1 to 3 mg/ml in methanol). The clear mixture was incubated at room temperature for about half an hour at room temperature, followed by dialysis (MWCO = 12,000) overnight against 2 L water. The dialysis solution was usually a clear solution. Trace amounts of paclitaxel particles were removed either by ultra-filtration (0.2 um filter) or by centrifugation (20 minutes at 20,800g and room temperature). The clear solution was stored at 4 °C or below.

Example 8

Preparation of Antisense oligonucleotide/CD-polymer complexes

Cyclodextrin PEG polymers (50 mg/ml) were mixed with a certain amount of a 21-mer-fluorescent labeled oligonucleotide in 20 mM Tris-HCl buffer (pH=7.4). The solutions were dried in a Speed-Vac, followed by reconstitution using the same amount of water. The DNA/polymer complexes in the solution were analyzed using 1% agarose gel in pH = 7.4 TAE buffer.

Table 3. Comparison of Paclitaxel or Oligonucleotide Loading by some of the co-polymers as compared with other available CD derivatives

Polymer	CD moiety / loading Polymer	Paclitaxel loading (mg / 50 mg polymer*)	Oligonucleotide (mg / 50 mg polymer*)
PEG-ss-CD	3.9	< 0.05	ND**
PEG-ss-AcCD	3.9	0.8	ND
PEG-C3-CD	4.8	<0.05	ND
PEG-C3-AcCD	4.8	~2.0	ND
PEG-L8-CD (A)	4.6	< 0.05	ND
PEG-L8-AcCD (A)	4.6	~2.6	ND
PEG-L8-CD (B)	5.9	<0.05	ND
PEG-L8-AcCD (B)	5.9	~2.9	ND
PEG-ss-DECD	1.5	0.4	~0.06
PEG-GFLG-DECD	2.5	3.0	~0.2
PEG-C3-DECD	2.6	<1.0	~0.15
PEG-L8-DECD	3.9	<1.0	~0.2
Controls			
HP-CD (from Sigma)		< 0.05	
$(SBE)_7$-CD (from Cydex)		< 0.05	
DM-CD (from Sigma)		~0.2 (at day 1)	
EP-CD (from Sigma)		< 0.05	

* Drug amount in 1.0 ml water or PBS in the presence of 50 mg of polymer or other CD derivatives.

** None detectable.

Example 9

Stability of Taxol/CD complexes in 50% serum or after 10 fold dilution in PBS:

(A) **Stability in 50% fetal bovine serum:** Taxol/PEG-L8-AcCD (2.0 mg / 50 mg in 1.0 ml PBS buffer) or Taxol/DMCD (0.5 mg/50 mg in PBS buffer) complex solutions were prepared as describe in method A of example 7. Fifty micro liters of the complex solutions were diluted with equal volume of fetal bovine serum respectively. Both mixtures were centrifuged at 20,8000 g at room temperature after incubation at room temperature for 2 hours, 21 hours, 49 hours and 144 hours, respectively. The Taxol concentration in each supernatant was quantified by measuring the UV absorption at 230 nm.

(B) **Stability after 10 fold dilution with PBS:** Taxol/PEG-L8-AcCD (2.0 mg / 50 mg in 1.0 ml PBS buffer) or Taxol/DMCD (0.5 mg/50 mg in PBS buffer) complex solutions were prepared as describe in example 10. Fifty micro liters of the complex solutions were diluted with 450 micro liter of PBS buffer, respectively. Both mixtures were centrifuged at 20,8000 g at room temperature after incubated room temperature for 2 hours, 21 hours, 49 hours and 144 hours. The Taxol concentration in each supernatant was quantified by measuring the UV absorption at 230 nm.

Table 4. Summary of the Stability Test of Paclitaxel/PEG-L8-AcCD and Paclitaxel /DMCD complexes in 50% Serum or after dilution with PBS

Time (hour)	Remaining paclitaxel % in diluted solution			
	PEG-L8-AcCD 50% Serum	PEG-L8-AcCD 10X PBS	DMCD 50% Serum	DMCD 10X PBS
0	100	100	100	100
2	100	100	100	86
21	97	100	100	13
49	92	99	100	
144	84	79	100	

Example 10

Release of paclitaxel from the Paclitaxel/PEG-L8-AcCD complexes and the cytotoxicity of the free co-polymers

The efficient release of the free paclitaxel from its PEG-L8-AcCD complex was confirmed by the cytotoxicity of the complexes. Similar IC_{50} values were obtained for both paclitaxel/PEG-L8-AcCD complex formulation (in this invention) and current commercial Paclitaxel/Cremophor formulation (Taxol, Bristol-Myers Squibb) in all three tested cell lines as determined by modified MTT assay as described below. But PEG-L8-AcCD alone showed no detectable cytotoxicity at the highest testing concentration while cremophor killed half of the cells at a concentration of about 0.5 mg/ml (Table 5):

1. Cells were plated at about 5,000 cells / well in 96-well plates in 0.1 ml medium and incubated at 37 °C for 24 hours;
2. Remove the old medium, add 80 ul of fresh media to each well;
3. Add 20 ul of sample solutions to each well (5X serially diluted, at least 8 concentrations for each sample)
4. The cells were incubated 3 or 4 days;
5. The media was removed. Added 80 ul of fresh media with 20 ul of MTS solution (Promega CellTiter 96 Aqueous One Solution Reagent #G358A). Incubate 37 °C for 2 to 4 hours;
6. Read absorbance at 490 nm on plate reader
7. Calculate the IC_{50} using cell free well as blank control and drug free well as 100% viability control.

Table 5. Comparison of the IC_{50}* of different Taxol formulations and carrier controls in three different cell lines

Formulations	IC_{50} (ng/ml)		
	Hela	HT1080	MCF7
Paclitaxel/PEG-L8-AcCD	3.0	2.0	2.0
Paclitaxel/Cremophor	3.0	4.0	2.0
Cremophor	500,000	500,000	500,000
PEG-L8-AcCD	> 10,000,000	>10,000,000	>10,000,000

* Concentration at which cells have 50% viability as evaluated by modified MTT assays:

Example 11

Hemolysis activity of the co-polymers and their possible biodegradation products:

To further investigate the cytotoxicity of our polymers and their possible biodegradation products, their hemolysis effects were tested on fresh human blood cells in comparison with commercial CD monomers as describe below. The degree of hemolysis was reported as a percentage of the total efflux of hemoglobin in distilled water (Table 6)

1.Red blood cells were isolated from whole human blood by centrifugation at 1000 g for 10 minutes.

2.The plasma was removed and the red blood cells re-suspended in normal buffed saline (PBS, 0.154 M sodium chloride and 0.01 M phosphate, pH = 7.4). The red blood cells were pelleted by centrifugation (1000g for 10 minutes).

3.Step 2 was repeated twice to remove the heme released from damaged cells.

4.The final pellet was diluted with PBS to give a hematocrit of approximately 12 (or 5%) as determined by centrifugal sedimentation.

5.2 ml of polymer or cyclodextrin solutions of a series of concentrations from 0 to 50 mg/ml in PBS buffer) equilibrated at 37°C in PBS buffer were equilibrated at 37 °C. To this was added 100 ul of a red blood cell suspension followed by mixing of the sample with gentle inversion. The samples were incubated for 30 minutes at 37° C.

6.The intact cells and cellular debris were pelleted by centrifugation at 1000 g for 5 min. The supernatant was analyzed spectrophotometrically at 543 nm for released heme.

Table 6. Comparison of Hemolysis activities of different PEG-CD polymers and their precursor monomers with commercial CD derivatives.

Comopmers or CD monomers	Hemolysis (HC_{50}, mM)
PEG-L8-AcCD	ND
PEG-L8-DECD	ND
PEG-L8-CD	ND
CD-L8-NH2	25
$(SBE)_7$-CD	ND
DM-CD	1.0
βCD	4.0
HPβCD	35

The above data show that the novel PEG-CD polymers of the present invention have great potential to be used as safe drug carriers for paclitaxel (Table 3,Table 4, Table 5 and Table 6). In the presence of 50 mg/ml of the polymers, the paclitaxel can be dissolved in water at a concentration of at least 2.2 mg/ml, which is more than a 10,000 fold increase in free paclitaxel water solubility, and at least 1,000 and 20 times better than that of hydroxylpropyl-β-cyclodextrin (HPCD) and methyl-β-cyclodextrin (DMCD), respectively, under similar conditions [Sharma et al. J

Pharm Sci, 84 (10), 1223-30 (1995)]. This dramatic solubility increase may due to a combination of at least the following three factors: 1) increased local concentration of CD moieties; 2) increased binding constant by cooperation the structure of paclitaxel has three phenyl groups around a large, fused taxane ring system); and 3) extra hydrophobic interactions outside the CD cavities.

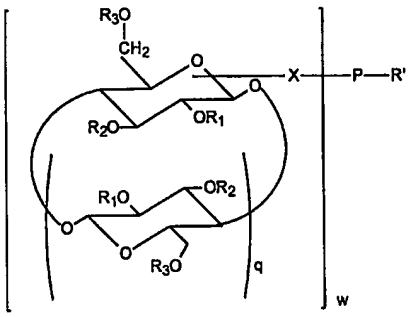
As expected, after being conjugated to PEG polymer, the toxicity of β-cyclodextrin was significantly reduced. No cytotoxicity was detected on all the cyclodextrin pendent PEG polymers as identified on MTT and hemolysis assays (Table 5 and Table 6). Even the monomer (building block) was much less toxic than natural β-cyclodextrin. On another hand, because the weight ratios of CD moieties in our current co-polymer were only less than 25% as determined by ^{1}H-NMR, the actual CD concentration in our experimental concentration (50 mg co-polymer / ml water) was less than 12.5 mg/ml. In another words, the weight ratio of cyclodextrin : Paclitaxel moiety was less than 6 : 1 in the current polymer complexes. Therefore, the co-polymers with non-biodegradable linkers are very safe drug carriers with very efficient drug release characteristics (Table 5). Additionally, the biodegradable linkage may also be acceptable as necessary to accelerate drug release.

The above Examples are presented for illustrative purposes only and are not intended, and should not be constructed to limit the invention in any manner. Various modifications of the compounds and methods of the invention may be made without departing from the spirit or scope thereof and it is to be understood that the invention is intended to be limited only as defined in the appended claims.

CLAIMS

We claim:

1. A cyclodextrin grafted biocompatible polymer having the formula 1:



wherein P is a biocompatible hydrophilic polymer backbone having a molecular weight range from 2,000 to 1,000,000 Daltons; R' is H or a targeting moiety; X is a linker having the formula

-Q-Z-Q'-

wherein Q is covalently bonded to the hydrophilic polymer chain either directly or by means of a pendant alkyl or other functional group and Q' is covalently bonded to the cylodextrin at the 2, 3 or 6 position thereby replacing either OR_1, OR_2 or OR_3 group respectively; Q and Q' are independently members selected from the group consisting of NR_4, S, O, CO, CONH, and COO; Z is a member selected from the group consisting of an alkylene disulfide, $[-(CH_2)_aS\text{-}S(CH_2)_a-]$, alkylene $[-(CH_2)_a-]$, alkylene oxide $(-[(CH_2)_aO]_b(CH_2)_a-)$, or a short chained peptide where a is an integer of 1 to 10 and b is an integer of 1 to 20; R_1, R_2, R_3 and R_4 are independently members selected from the group consisting of H, alkyl ($C_{n'}H_{2n'+1}$), alkenyl ($C_{n'+1}H_{2(n'+1)-1}$) or acyl ($C_{n'}H_{2n'+1}CO$) where n' is an integer of 1 to 16; q is an integer of 5, 6 or 7; and w is an integer such that each polymer backbone contains between 1.5 and 30 cyclodextrin moieties per 20 KD of polymer backbone.

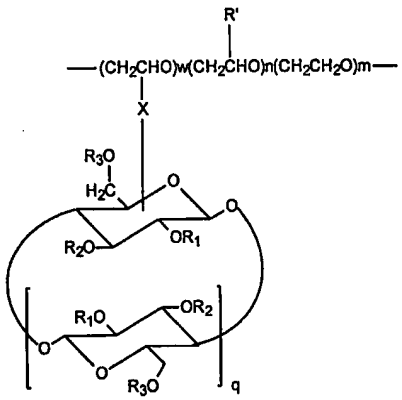
2. The cyclodextrin grafted biocompatible polymer of Claim 1 wherein the biocompatible polymer is a member selected from the group consisting of

polyethylene glycol (PEG), N-(2- hydroxypropyl)methacrylamide polymer (HPMA), polyethylenimine (PEI), polylysine, derivatives thereof and polymers thereof.

3. The cyclodextrin grafted biocompatible polymer of Claim 2 wherein each polymer backbone contains between 2 and 15 cyclodextrin moieties per 20 KD of polymer backbone.

4. The cyclodextrin grafted biocompatible polymer of Claim 3 wherein the biocompatible polymer has a molecular weight of between about 5,000 and 70,000.

5. A cyclodextrin grafted biocompatible polymer having the formula 2



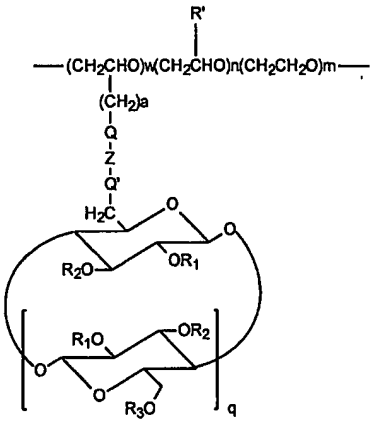
wherein R' is H or a targeting moiety; X is a linker having the formula

-Q-Z-Q'-

wherein Q is covalently bonded to the hydrophilic polymer chain either directly or by means of a pendant alkyl or other functional group and Q' is covalently bonded to the cylodextrin at the 2, 3 or 6 position thereby replacing either OR_1, OR_2 or OR_3 group respectively; Q and Q' are independently members selected from the group consisting of NR_4, S, O, CO, CONH, and COO; Z is a member selected from the group consisting of an alkylene disulfide, $[-(CH_2)_aS\text{-}S(CH_2)_a-]$, alkylene $[-(CH_2)_a-]$, alkylene oxide $(-[(CH_2)_aO]_b(CH_2)_a-)$, or a short chained peptide where a is an integer of 1 to 10 and b is an integer of 1 to 20; R_1, R_2, R_3 and R_4 are independently members selected from the group consisting of H, alkyl ($C_{n'}H_{2n'+1}$), alkenyl ($C_{n'+1}H_{2(n'+1)-1}$) or

acyl ($C_{n'}H_{2n'+1}CO$) where n' is an integer of 1 to 16; q is an integer of 5, 6 or 7; w is an integer such as to provide between 2 and 15 cyclodextrin units per 20 KD PEG backbone chain, and m and n are integers sufficient that when combined with w they represent a polyethylene oxide polymeric chain having the molecular weight of 5,000 to 70,000 with the proviso that monomeric units on the biocompatible polymer backbone containing the grafted cyclodextrin units represented by w do not have to be consecutively joined but may be randomly or uniformly distributed along polymer backbone.

6. A cyclodextrin grafted biocompatible polymer having the formula 3



wherein R' is H or a targeting moiety; Q is covalently bonded to the hydrophilic polymer chain either directly or by means of a pendant alkyl or other functional group and Q' is covalently bonded to the cylodextrin at the 2, 3 or 6 position thereby replacing either OR_1, OR_2 or OR_3 group respectively; Q and Q' are independently members selected from the group consisting of NR_4, S, O, CO, CONH, and COO; Z is a member selected from the group consisting of an alkylene disulfide, $[-(CH_2)_aS-S(CH_2)_a-]$, alkylene $[-(CH_2)_a-]$, alkylene oxide $(-[(CH_2)_aO]_b(CH_2)_a-)$, or a short chained peptide where a is an integer of 1 to 10 and b is an integer of 1 to 20; R_1, R_2, R_3 and R_4 are independently members selected from the group consisting of H, alkyl ($C_{n'}H_{2n'+1}$), alkenyl ($C_{n'+1}H_{2(n'+1)-1}$) or acyl ($C_{n'}H_{2n'+1}CO$) where n' is an integer of 1 to 16; q is an integer of 5, 6 or 7; w is an integer such as to provide between 2 and 15 cyclodextrin units per 20 KD PEG backbone chain, and m and n are integers sufficient that when combined with w they represent a polyethylene oxide polymeric chain having the molecular weight of 5,000 to 70,000 with the proviso that

monomeric units on the hydrophilic polymer chain containing the cyclodextrin units represented by w do not have to be consecutively joined but may be randomly or uniformly distributed along polymer chain.

7. The cyclodextrin grafted biocompatible polymer of one of the Claims 4 to 6 where Q is C(O)NH, Q' is NR_4 and a is 2.

8. The cyclodextrin grafted biocompatible polymer of one of the Claims 4 to 6 where Z is $-(CH_2)_2S-S(CH_2)_2-$; R_4 is C_2H_5, $R_1$is C_2H_5, R_2 is H and R_3 is C_2H_5.

9. A composition comprising a cyclodextrin grafted biocompatible polymer of one of the Claims 1 to 6 and an active agent.

10. The composition of Claim 19 wherein the active agent is a hydrophobic drug, a protein or peptide drug, a nucleic acid or an oligo nucleotide

11. The composition of Claim 11 wherein the active agent is paclitaxel.

Figure 1

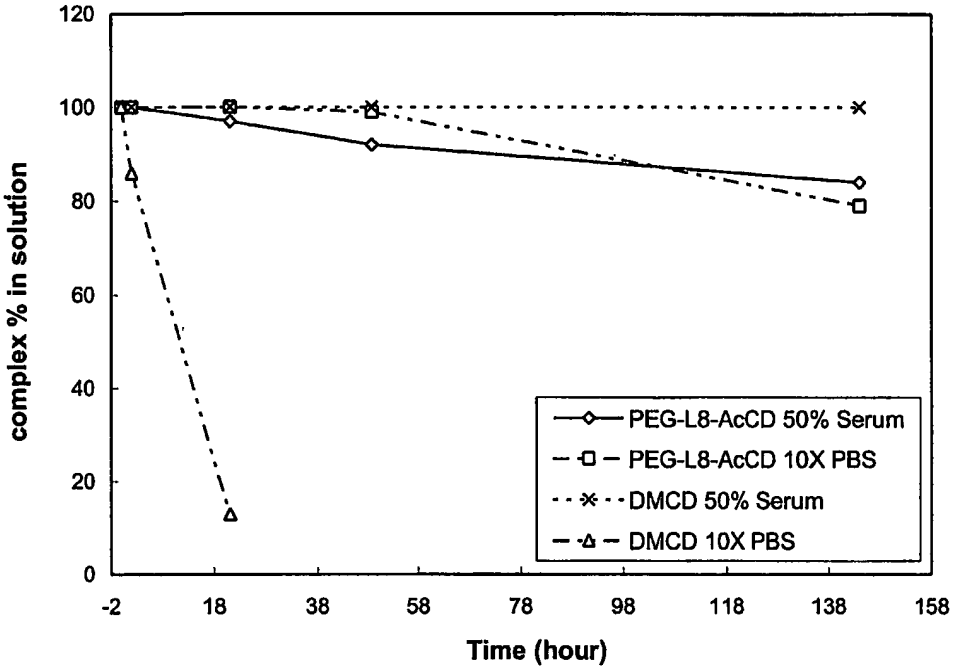

120
100
80
60
40
20
0
complex % in solution
-2
18
38
58
78
98
118
138
158
Time (hour)
PEG-L8-AcCD 50% Serum
PEG-L8-AcCD 10X PBS
DMCD 50% Serum
DMCD 10X PBS

Figure 2

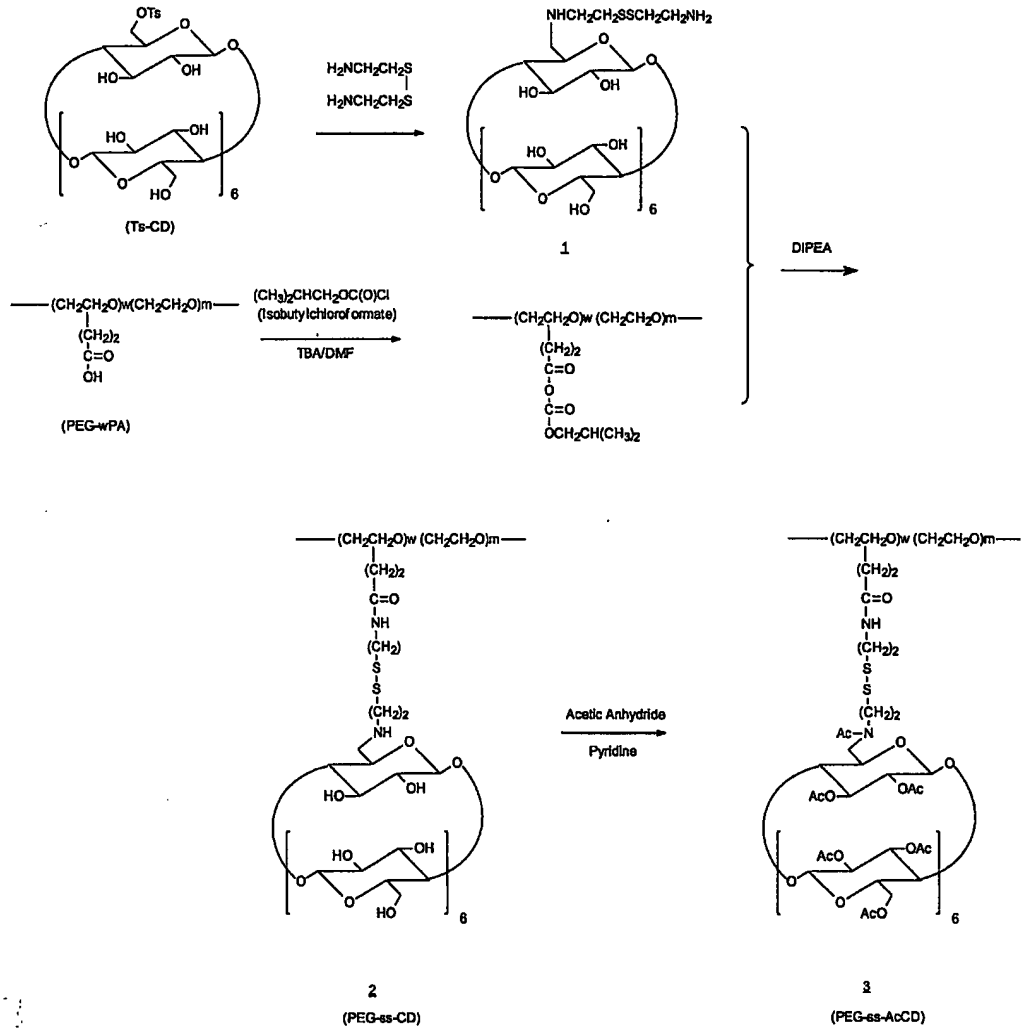

OTs
HO
OH
HO
OH
HO
6
(Ts-CD)
H2NCH2CH2S
H2NCH2CH2S
NHCH2CH2SSCH2CH2NH2
1
(CH2CH2O)w(CH2CH2O)m
(CH2)2
C=O
OH
(PEG-wPA)
(CH3)2CHCH2OC(O)Cl
(Isobutylchloroformate)
TBA/DMF
(CH2CH2O)w (CH2CH2O)m
O
OCH2CH(CH3)2
DIPEA
NH
(CH2)
S
(CH2)2
2
(PEG-ss-CD)
Acetic Anhydride
Pyridine
Ac-N
AcO
OAc
3
(PEG-ss-AcCD)

Figure 3

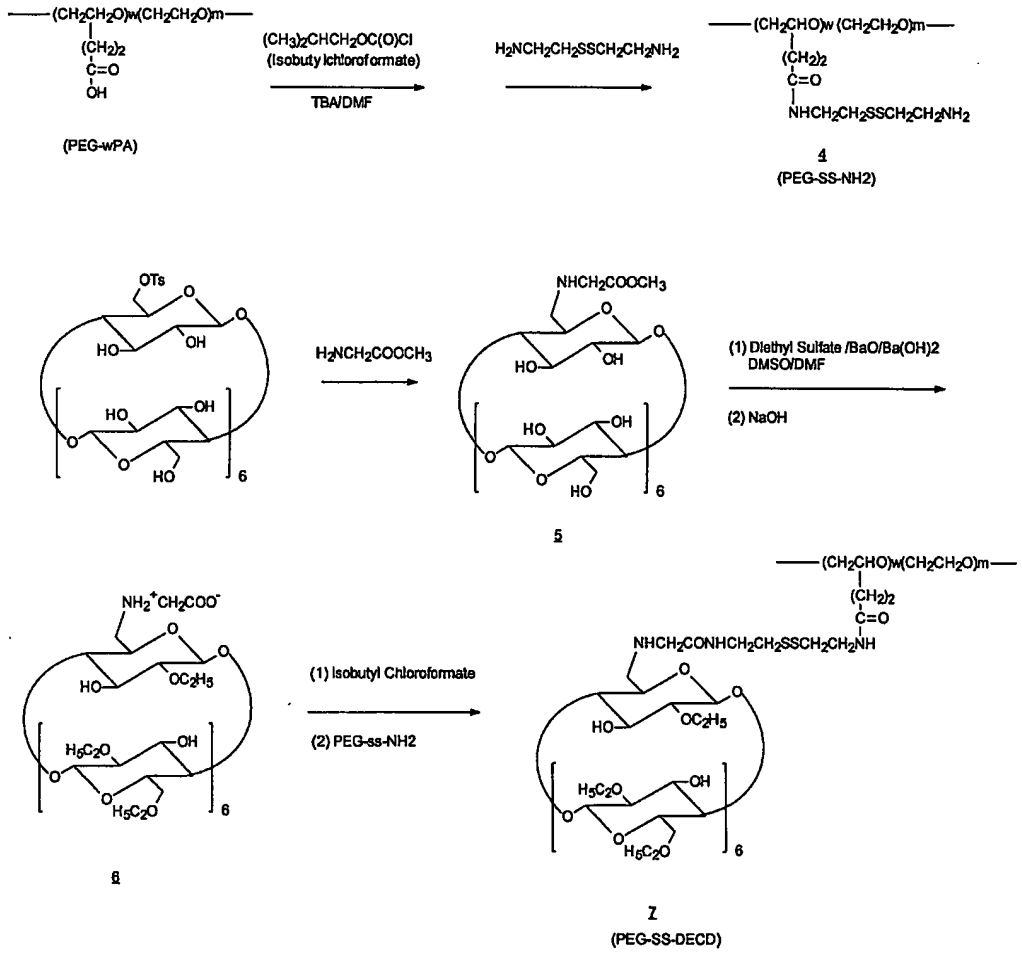
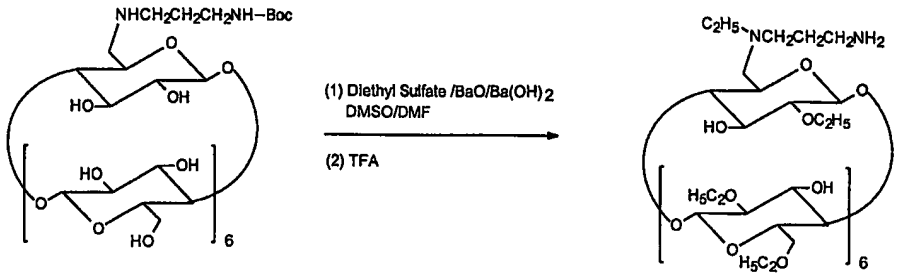

(PEG-wPA)
(CH3)2CHCH2OC(O)Cl
(Isobutyl chloroformate)
TBA/DMF
H2NCH2CH2SSCH2CH2NH2
4
(PEG-SS-NH2)
H2NCH2COOCH3
(1) Diethyl Sulfate /BaO/Ba(OH)2
DMSO/DMF
(2) NaOH
5
6
(1) Isobutyl Chloroformate
(2) PEG-ss-NH2
7
(PEG-SS-DECD)

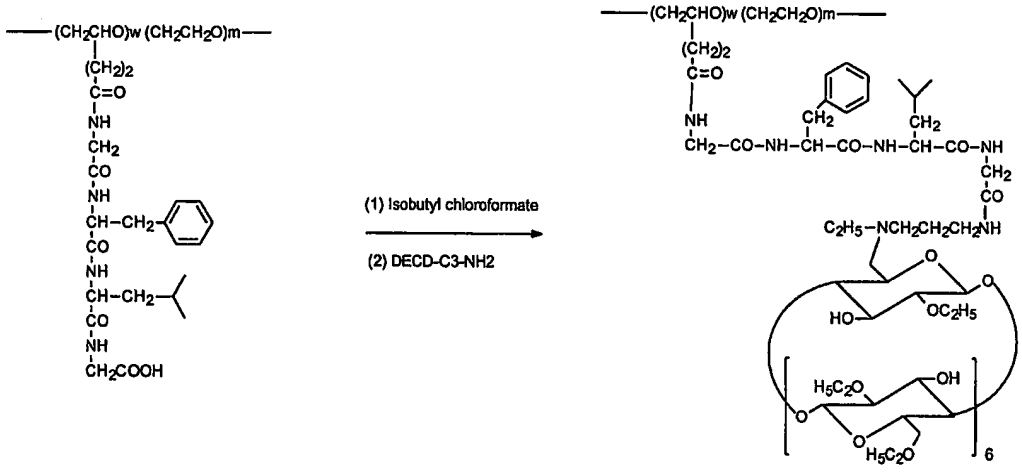
Figure 4



8

9

(DECD-C3-NH2)



10

(PEG-GFLG)

11

(PEG-GFLG-DECD)

Figure 5

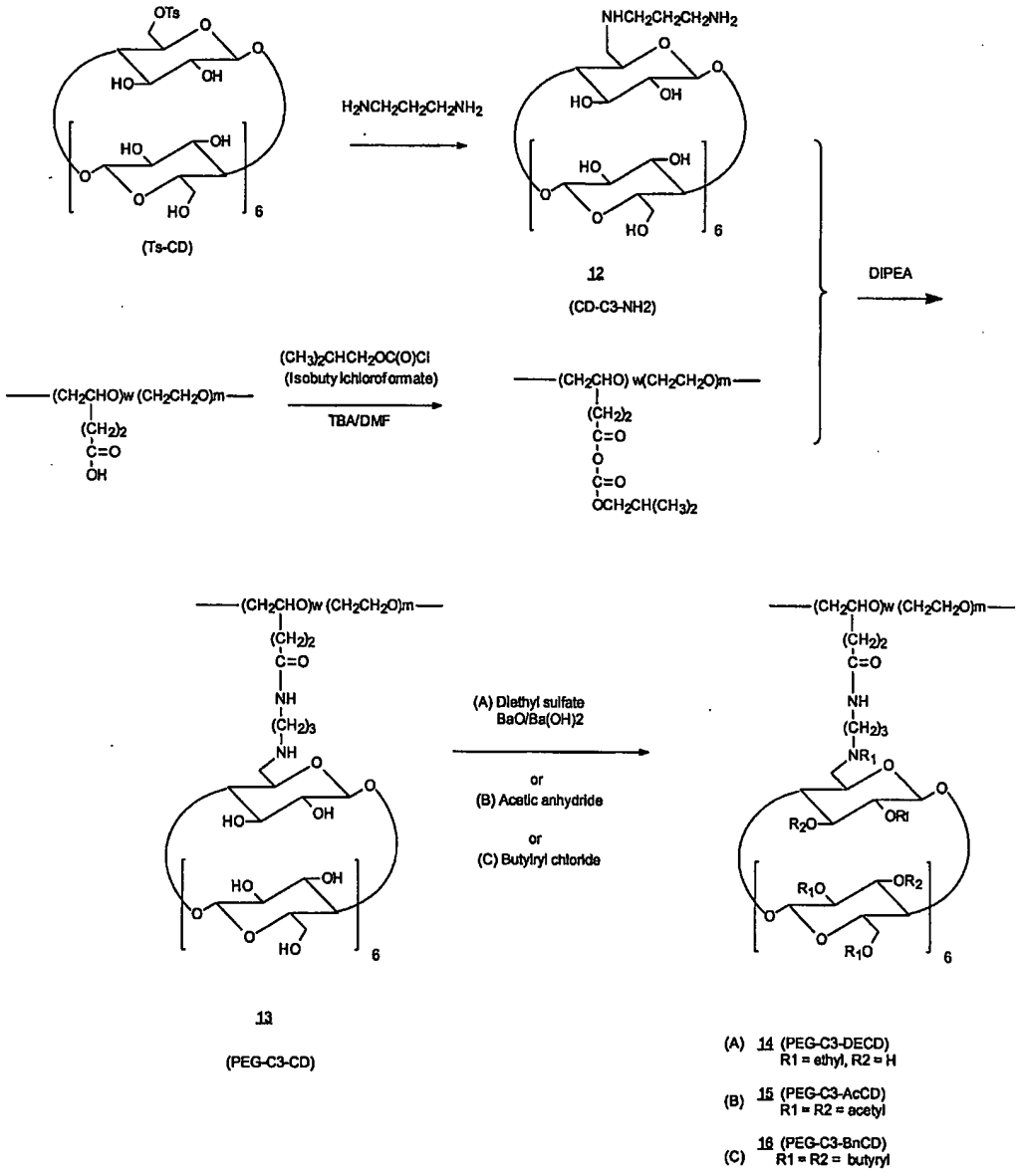
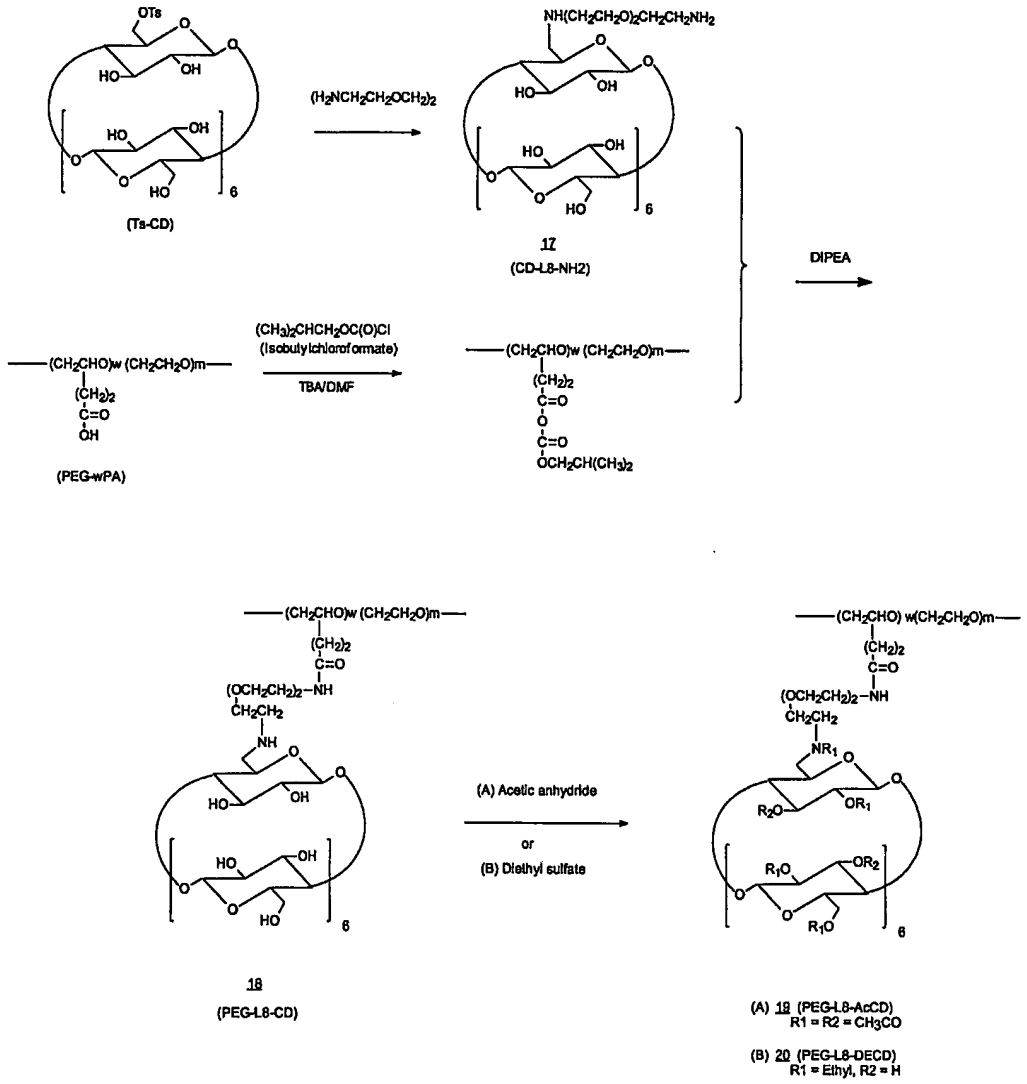

OTs
HO
OH
HO
OH
HO
6
(Ts-CD)
H2NCH2CH2CH2NH2
NHCH2CH2CH2NH2
HO
OH
HO
OH
HO
6
12
(CD-C3-NH2)
DIPEA
(CH2CHO)w (CH2CH2O)m
(CH2)2
C=O
OH
(CH3)2CHCH2OC(O)Cl
(Isobutylchloroformate)
TBA/DMF
(CH2CHO) w(CH2CH2O)m
(CH2)2
C=O
O
C=O
OCH2CH(CH3)2
(CH2CHO)w (CH2CH2O)m
(CH2)2
C=O
NH
(CH2)3
NH
HO
OH
HO
OH
HO
6
13
(PEG-C3-CD)
(A) Diethyl sulfate
BaO/Ba(OH)2
or
(B) Acetic anhydride
or
(C) Butyryl chloride
(CH2CHO)w (CH2CH2O)m
(CH2)2
C=O
NH
(CH2)3
NR1
R2O
OR1
R1O
OR2
R1O
6
(A) 14 (PEG-C3-DECD)
R1 = ethyl, R2 = H
(B) 15 (PEG-C3-AcCD)
R1 = R2 = acetyl
(C) 16 (PEG-C3-BnCD)
R1 = R2 = butyryl

Figure 6


OTs
HO
OH
6
(Ts-CD)
(H2NCH2CH2OCH2)2
NH(CH2CH2O)2CH2CH2NH2
17
(CD-L8-NH2)
DIPEA
(CH2CHO)w (CH2CH2O)m
(CH2)2
C=O
OH
(PEG-wPA)
(CH3)2CHCH2OC(O)Cl
(Isobutylchloroformate)
TBA/DMF
O
OCH2CH(CH3)2
(OCH2CH2)2–NH
CH2CH2
NH
18
(PEG-L8-CD)
(A) Acetic anhydride
or
(B) Diethyl sulfate
NR1
R2O
OR1
R1O
OR2
(A) 18 (PEG-L8-AcCD)
R1 = R2 = CH3CO
(B) 20 (PEG-L8-DECD)
R1 = Ethyl, R2 = H